SIRC

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JUL 1 9 2004

July 16, 2004

Dr. C. W. Jameson
National Toxicology Program
Report on Carcinogens
79 Alexander Drive, Building 4401, Room 3118
P.O. Box 12233
Research Triangle Park, NC 27709

Re: Nomination of Styrene for Review for 12th Report on Carcinogens

Dear Dr. Jameson:

Enclosed are comments from the Styrene Information and Research Center (SIRC) on the nomination of styrene for review for the 12th Report on Carcinogens. These hard copies are duplicates of two documents submitted to your office by e-mail on Friday, July 16. Please contact me at 703-741-5010 if you have any questions.

Sincerely,

Jack Snyder
Executive Director

July 16, 2004

Dr. C. W. Jameson
National Toxicology Program
Report on Carcinogens
79 Alexander Drive, Building 4401, Room 3118
P.O. Box 12233
Research Triangle Park, NC 27709

Re: Nomination of Styrene for Review for 12th Report on Carcinogens

Dear Dr. Jameson:

The *Federal Register* of May 19, 2004, contained a notice that NTP would evaluate 21 chemicals in 2004 and 2005 for possible inclusion in the 12th edition of the Report on Carcinogens (RoC), and requested comments within 60 days. 69 Fed. Reg. 28940 (May 19, 2004). Styrene was included in the list based on the 2002 conclusion by the International Agency for Research on Cancer (IARC) that the human data provided "limited" evidence of carcinogenicity, along with its previous IARC conclusion of "limited animal data."

For the reasons detailed below and in the attached review document, the Styrene Information and Research Center, Inc.[1] (SIRC) believes that styrene does not meet NTP's criteria for listing in the RoC, and therefore recommends that styrene not be included in the 12th RoC.

In this cover letter, we briefly address the inaccuracies in the IARC description of key human studies that affect the interpretation of the individual studies and the overall weight of the evidence. To substantiate these criticisms, the attached review document provides a more comprehensive evaluation of the styrene database of human and animal studies, and mode of action data.

A. IARC Interpretation of Animal Data

We agree with IARC that the animal data provide only limited evidence of carcinogenicity based upon:
- The clear lack of carcinogenicity in rats, as supported by numerous studies in which rats were given styrene by a variety of routes and
- Findings of only lung tumors in mice, particularly when exposed by inhalation.

Extensive mode of action data indicate that the mouse lung tumors are the result of cytotoxicity caused by *in situ* metabolism of styrene and are not relevant for human risk assessment. Since the time of the IARC review, fairly strong evidence indicates that ring-oxidized products of styrene are responsible for the cytotoxicity and styrene-7,8-oxide plays a minor role, if any. These ring-oxidized products are generated by CYP2F2 in the mouse lung. In contrast, the metabolism of styrene, including ring oxidation, in the human lung is extremely low and no activity of the human CYP2F1 to styrene has been detected. This provides further evidence that the mouse lung tumors are not relevant for human risk assessment.

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[1] The Styrene Information and Research Center's (SIRC's) mission is to evaluate existing data on potential health effects of styrene, and develop additional data where it is needed. SIRC has gained recognition as a reliable source of information on styrene and helping ensure that regulatory decisions are based on sound science. For more information, visit http://www.styrene.org/.

B.   IARC Interpretation of Human Data

1.   Synopsis of IARC Reviews

In 1987, IARC changed the classification of styrene from category 3 (not classifiable) to 2B (possible carcinogen). While IARC considered the human data to be inadequate and the animal data to be limited, it changed styrene's classification based on genotoxicity and metabolism to styrene oxide. This classification and data descriptors were reaffirmed in 1994.

In 2002, styrene remained in Group 2B, but the classification was based on limited human data, limited animal data and no upgrade due to mode of action data. The IARC Epidemiology Subgroup for that review debated until the last minute whether to recommend "inadequate" or "limited" for the human data, finally opting to recommend "limited." The plenary IARC Working Group, however, was not in full agreement with this recommendation, and only accepted the "limited" characterization by a vote of 16-8. In addition, there are a number of key analytical points not included in the IARC summary that NTP should consider.

2.   Critique of IARC's Interpretation of Human Data

While IARC rightfully acknowledged that studies in the reinforced plastics workers are the most relevant for evaluating styrene carcinogenic potential in humans, the NTP nominations document cites data from monomer, polymer and styrene-butadiene rubber (SBR) manufacture as corroborating evidence. We will deal with these latter studies first:

> **a. Monomer, polymer and styrene-butadiene rubber (SBR) manufacture** - IARC reports that two studies of workers in the United States and United Kingdom involved in styrene monomer and polystyrene production found a weak association between styrene exposure and lymphatic and hematopoietic cancers. However, in the U.S. study (Ott et al., 1980, Bond et al., 1992), the authors reported that the risk among workers in high-exposure groups was unremarkable, and there was no pattern of risk with regard to year of first exposure or latency. Therefore, this does *not* indicate an association of styrene with increased cancer.
>
> In the second study, Hodgson and Jones (1985) reported a significant increase in lymphoma (not leukemia) among 622 workers involved in the production of styrene monomer, polymerization and manufacture of finished products. However, workers were exposed to many other chemicals in addition to styrene. Significantly, no measure of styrene exposure was taken and no attempt was made to determine if the increase was "associated with styrene exposure."
>
> In the SBR cohort, most recently studied by Delzell and coworkers, IARC correctly reports that increased leukemia was more strongly correlated with butadiene exposure than styrene exposure, but concludes that concomitant exposures to styrene and butadiene "makes it difficult to disentangle the effects of these two exposures." Regarding leukemia, Delzell and coworkers (2001) concluded:
>
> "After further adjusting each agent-specific set of RRs [Relative Risks] for the other two agents, a positive but imprecise relation remained for BD [butadiene] and DMDTTC [dimethyldithiocarbamate] but not for STY [styrene]. . . . . BD and DMDTTC, but not STY,

were positively associated with leukemia in multivariate analyses. The independent effect of each agent was difficult to evaluate because of correlations with other agents and imprecision."

Thus, although workers were often exposed to both butadiene and styrene, the authors indicate that when butadiene exposure was considered, styrene was not a factor in leukemia.

Although IARC portrayed the data in the styrene manufacturing / polymerization and SBR industries as corroborating increased cancer risks from styrene, the studies do *not* provide evidence of increased cancer risk from styrene.

b. Reinforced plastics manufacture - The IARC treatment of the studies of reinforced plastics workers merits further consideration. In its evaluation, IARC ignored the National Institute for Occupational Safety and Health study by Okun *et al.*, (1985), which showed no increase in lymphatic or hematopoietic cancer. This study has since been updated by the addition of 21 years of follow-up (Ruder *et al.*, 2004). While it is still the smallest of the three independent studies (5201 workers), it has the longest follow-up (average 26 years). There still was no increase in lymphatic or hematopoietic cancers.

IARC acknowledges that the other U.S. cohort study (Wong, 1990, Wong *et al.*, 1994) showed no excess in lymphatic or hematopoietic cancers relative to time since first exposure, duration of exposure or cumulative exposure. They questioned the findings because: "The inclusion of two correlated indices of styrene exposure in the regression models may have artificially reduced the coefficients of both." But, Wong clearly states that he performed two proportional hazard analyses: one with only cumulative exposure and one with cumulative exposure and length of exposure. The result of both models was the same. Thus, this study of 15,826 workers with an average of 19.5 years follow-up provides no evidence of styrene-related cancer increase and was incorrectly cited by IARC.

IARC characterized the Kogevinas et al. (1994) study as showing that "the risk of lymphatic and hematopoietic neoplasms was significantly increased among exposed workers after more than 20 years since their first exposure to styrene, and increased with increasing intensity of exposure, but not with increasing cumulative exposure to styrene." IARC commented that the lack of a response relative to cumulative exposure might have been unreliable because cumulative exposure was under-estimated, especially in the Danish sub-cohort. Because of the way average level of exposure ("intensity") was calculated, if the duration of exposure was unreliable, then the average level of exposure was also unreliable.

The increases in the Kogevinas study are almost entirely contributed by the Danish sub-cohort, which was drawn from the Kolstad et al. (1994) study. The Danish sub-cohort contributed 49% of the overall lymphatic and hematopoietic cancers and 59% of the leukemias, while contributing only 39% of the cohort members and 32% of the person-years. Because the Epidemiology Subgroup at the IARC Working group on styrene stated that the Kolstad study was the key positive study to justify upgrading the classification of the human data from "insufficient" to "limited," a careful review of this study is clearly warranted. In this cohort there was no attempt to ascertain if any of the cases of cancer were among the minority of workers that were involved in lamination, versus those not involved in any aspect of reinforced plastics manufacture. Furthermore, there are a number of issues with cohort assembly, employment duration, exposure definition and relationships of cancers to styrene exposure, which are detailed further in our attached review. While Kolstad et al. (1994) found

a significant excess of leukemia among workers hired before 1970 when a 10-year lag time was applied, this phenomenon was only observed among short-term workers (less than one year employment) with uncertain and undocumented exposure to styrene. A more extensive assessment of both the Kogevinas and Kolstad studies is included in the attached review document.

C. Comments on "Summary NTP Nomination for Review" for Styrene

1. Genetic and Genetic Related Effects

In regard to the minority of studies of genetic damage in workers exposed to styrene that were positive, the NTP Nomination Summary states (p. 5) that "Most positive studies were from the reinforced plastics industries, where exposures were the highest." While not stated explicitly, this implies that the increases were most likely due to styrene exposure. It should be noted that nearly all of the negative studies were also from workers in the reinforced plastics industry.

Secondly the summary says "Styrene induced sister chromatid exchange and chromosome aberrations in rodents *in vivo*." While nearly all studies that have examined the effect of styrene on sister chromatid exchange have reported a small increase (up to 20% greater than control), only 1 of 9 studies that have investigated chromosomal aberrations in rodents has reported an increase.

Thirdly, the Summary says "...styrene induced mutations in some but not all studies of bacterial strains with metabolic activation." While a true statement, it is certainly misleading. In fact, the authors reported increased mutation frequency in 0 of 12 studies of *Salmonella* TA98; 1 of 12 studies in TA100; 4 of 12 studies in TA1535; 0 of 10 studies in TA1537; and 0 of 10 studies in TA1538. Of most significance are the studies of Dunkel et al. (1985) that tested styrene against all five strains in four independent laboratories: all four laboratories reported no increase in mutations. A complete review of the genetic toxicity data on styrene is included in the accompanying review.

2. Other Relevant Data

The NTP Summary indicates that conversion of styrene oxide by epoxide hydrolase to mandelic acid, etc., is most important in humans, but that reaction of styrene oxide with glutathione is most important in rodents. That statement is incorrect. In humans nearly all styrene is excreted as products from the epoxide hydrolase pathway (Johanson et al., 2000). In rodents, 55-75% of styrene is also metabolized via the epoxide hydrolase pathway (Sumner et al., 1995). Glutathione conjugates account for 20-30% of styrene urinary excretion products in rats and mice.

Most importantly, this summary completely ignored the extensive mode of action database for styrene-induced mouse lung tumors, which indicates that the tumors are the result of *in situ* metabolism of styrene in mouse lung by CYP2F2, as summarized in Cruzan et al. (2002). IARC concluded that "it is likely that the proposed mechanism involving metabolism of styrene to styrene 7,8-oxide in mouse lung Clara cells is not operative in human lungs to a biologically significant extent." Subsequent to the 2002 article, additional evidence has developed that CYP2F2 is also responsible for ring oxidation of styrene and that it is most likely that ring oxidized metabolites are responsible for the cytotoxicity and tumor formation. These data are assessed in the accompanying review.
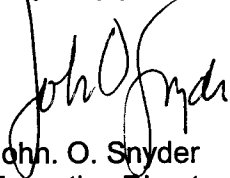
D.   Conclusion

Overall, the human data from 12 cohort mortality studies do not present any convincing evidence that styrene causes increased cancer in humans. Among more than 61,000 reinforced plastics workers in three studies, which represent the workers most highly exposed to styrene, there is no consistent increase in any cancer, specifically with lymphatic and hematopoietic cancers. In the Kolstad study, increased leukemia was found among short-term, but not long-term workers; in addition, it is not possible from this study to determine if any of the leukemia cases were actually exposed to styrene. Increased cancers in monomer, polymer and SBR workers are not attributed to styrene. While there are some increased cancer deaths among workers in industries that use styrene, there is no evidence that these increases are caused by styrene exposure.

The results of 13 chronic studies of styrene in rats and mice indicate styrene induces mostly benign tumors very late in the study in a single species (mouse), at a single site in an organ with a high background incidence (lung). Extensive mode of action data indicate mouse-lung-specific metabolism of styrene is responsible, and that this metabolism does not take place to a measurable extent in human lung. Accordingly, neither the human data nor the animal data for styrene meet NTP's criteria for listing in the Report on Carcinogens, and SIRC believes that styrene therefore is not an appropriate candidate for listing therein.

We very much appreciate your careful consideration of the enclosed document. Please contact me if you have any questions, or if you or your associates would like to discuss the material we have provided.

Very truly yours,

John. O. Snyder
Executive Director
Styrene Information & Research Center

Enclosure: SIRC Comments on the NTP Nomination of Styrene for the 12th Report on Carcinogens

Note: Please see attached document for literature references on studies cited in this letter.

STYRENE INFORMATION AND RESEARCH CENTER (SIRC)

COMMENTS ON THE NATIONAL TOXICOLOGY PROGRAM

NOMINATION OF

STYRENE

FOR THE 12TH REPORT ON CARCINOGENS

July 16, 2004

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SIRC COMMENTS ON THE NTP NOMINATION OF STYRENE FOR THE 12TH REPORT ON CARCINOGENS

The National Toxicology Program (NTP) nominated the chemical styrene for inclusion in the 12th Report on Carcinogens. 69 Fed. Reg. 28940 (May 19, 2004). The nomination was based on a review by the International Agency for Research on Cancer (IARC) that characterized the human data as demonstrating "limited" evidence of carcinogenicity (Vol. 82, 2002). However, applying NTP's listing criteria, a review of the current scientific literature on styrene demonstrates that is *not* an appropriate candidate for inclusion in the Report on Carcinogens.

1. Analysis of Tumor Data

1.1. Epidemiology

A number of epidemiologic studies of workers exposed to styrene involving the production of styrene/polystyrene (PS), styrene-butadiene latex rubber (SBR), and reinforced plastics and composites (RPC) have been published. These are listed in detail in Appendix A. Because these are very different industries with different styrene exposures and confounding exposures, the studies are reviewed according to industry segment. The same or different primary authors have updated several studies, while some have been combined into larger cohorts. Emphasis in this review has been placed on the updated or combined study reports.

This review focuses on the key study attributes and findings pertaining to the evaluation of cancer causality, including study design, reference group, potential sources of bias and confounding, and exposure classification. These are important elements to be considered in performing a weight of evidence evaluation regarding styrene's potential carcinogenicity. In addition, key criteria among the generally accepted criteria for causality were given particular attention in this review, namely: (1) strength of association, (2) consistency, (3) specificity, (4) temporality, (5) biologic gradient (dose-response), (6) biologic plausibility, and (7) coherence.

The manufacture of styrene and most styrenic polymers occurs in closed vessels and thus exposure to styrene in this industry is quite low. Time-weighted average exposures to styrene are less than 5 parts per million (ppm) (Miller et al., 1994). In addition, workers are potentially exposed to a number of other chemicals, including benzene, ethylbenzene, and acrylonitrile. Exposures to styrene in the SBR industry are also low, with time-weighted average exposures of approximately 3.5 ppm (Matanoski et al., 1993). Exposures of RPC workers are considerably greater, with past average exposures in many jobs being greater than 100 ppm (Wong, 1990; Kogevinas et al., 1993). The greatest potential exposure to styrene was through the inhalation of vapors during manual spray-up or lay-up operations and curing, and dermal contact during hand lamination with resin that contained about 40 percent styrene monomer.

1.1.1. Styrene Manufacture and Polymerization

Frentzel-Beyme et al. (1978) provided the first epidemiological assessment within this industry segment among 1,960 workers in Germany employed for at least one month between 1931 and 1976 (20,138 person-years of follow-up). Airborne concentrations of styrene measured in the mid-1970s were all less than 10 ppm, and most were less than 1 ppm (Theiss et al., 1978). Historical levels were not provided. A total of 73 deaths were observed compared to 96.5 expected based on mortality rates in two local regions during 1970 to 1975. There was one death attributed to LH cancer compared to less than one expected. A proportionate mortality ratio (PMR) analysis was also performed. Similar small numbers of deaths were observed for lung, kidney (urinary system), and pancreatic cancers. No unusual excess mortality was observed for any specific cause of death.

Nicholson et al. (1978) also reported the results of a small study of 560 men with at least 5 years employment at a plant in the U.S. that manufactured styrene monomer and polystyrene. Other plant activities included the production of styrene-butadiene latex and pilot plant operations for research and development work on styrene polymers. Airborne levels of styrene measured in 1974 in departments with the highest exposures ranged from 5 to 20 ppm, while in low-exposure areas they were less than 1 ppm. Other chemicals handled included benzene, ethylbenzene, toluene, xylene, and 1,3-butadiene. The 560 cohort members were active employees in May 1960, and were followed through 1975. Expected numbers of death for the major disease categories were calculated based on national rates, as well as for lung cancer, leukemia, and lymphoma. A total of 83 deaths were observed compared to 106.4 expected (17 total cancers compared to 21.0 expected). There was one death each from leukemia and lymphoma, which was about as expected. A total of six lung cancer deaths were observed versus seven expected. The study also reported on a subsequent review of "randomly collected death certificates" among persons at the plant who had worked for six months or longer. Among 444 deaths identified, seven were leukemias, three were Hodgkin's disease, and two were lymphomas. No corresponding expected numbers of death were presented.

Ott et al. (1980) reported on an initial cohort study of 2,904 male employees who worked for at least 1 year between 1937 and 1970 in four plants engaged in the manufacture and finishing of styrene monomer, production of styrene-butadiene latex, polymerization, coloring and extrusion of styrene-based products, and styrene research and development. Mortality patterns were evaluated through 1975 and compared to rates in the general U.S. population. An 11-year follow-up study of this cohort was reported by Bond et al. (1992). Among employees in monomer production, time-weighted average exposures to styrene were estimated from surveys during 1962 to 1976 to be less than 10 ppm. Exposures during the production of styrene-butadiene latex were estimated to range from 4-7 ppm in 1973. Measurements in relation to polymerization, coloring, and resin extrusion were in the range of 5-9 ppm before 1970, and 1-4 ppm during the 1970s. In addition to styrene, workers were potentially exposed to a number of other chemicals, including benzene, ethylbenzene, acrylonitrile, 1,3-butadiene, colorants, dyes, and antioxidants. A small subset of workers also worked with arsenicals, vinyl chloride and asbestos. Age-, race- and calendar-specific U.S. mortality rates were used to calculate expected numbers of death.

Over the combined follow-up period, a total of 687 deaths were observed compared to 903.3 expected (SMR 76, 95% CI 70-82). The total number of deaths due to malignant neoplasms was similarly less than expected (SMR 81, 95% CI 69-95). There was an increase in total LH cancer (SMR 144, 95% CI 95-208), which was mainly due to increases in Hodgkin's disease (SMR 222, 95% CI 71-518) and multiple myeloma (SMR 184, 95% CI 74-380). Deaths from leukemia were slightly elevated (SMR 118, 95% CI 54-224). Based on further analyses by work area and duration of employment, these increases were not observed among employees considered to have the highest exposures to styrene or among long-term employees. Instead, the highest risks were seen among professional employees and workers with short-term exposure to extrusion fumes, solvents and colorants. Lung cancer deaths were not elevated in the total cohort (SMR 81, 95% CI 61-105) or among any of the four major exposure groups. The same was true for kidney cancer (SMR 98, 95% CI 32-230) and pancreatic cancer (SMR 49, 95% CI 16-113). Breast cancer was not presented since only males were included in the analysis. No other cancer excesses were reported.

Hodgson and Jones (1985) studied both mortality and cancer incidence among 622 men who worked at least one year between 1945 and 1978 in a plant in the U.K. engaged in the production, polymerization and processing of styrene. No exposure measurements were available, although the levels were likely "well below the hygienic standard of 100 ppm." According to the authors, the plant was part of a much larger general chemical works, and workers commonly moved from one process to another. Deaths from major cause categories and selected cancers, as well as the incidence of total cancer and LH cancer, were compared to expected numbers based on rates for males in England and Wales. A total of 34 deaths were observed compared to 43.1 expected. There were 10 total cancer deaths (compared to 10.9 expected); 5 were due to lung cancer (4.2 expected) and 3 were due to lymphomas (0.56 expected). There were no deaths from leukemia. Breast, kidney and pancreatic cancers were not reported. Based on the analysis of cancer incidence, there were a total of 4 incidence cases of LH cancer observed (3 lymphomas; 1 chronic lymphatic leukemia) compared to 1.6 expected (SIR 250, 95% CI 68-640). A further review of work histories among the four cases revealed exposures to acrylonitrile, benzene, pitch, polyvinylchloride, dyestuffs, antioxidants, and ethylene oxide in addition to styrene. There was no apparent association between length of service in the styrene-exposed jobs and the incidence of LH cancer. The only other finding was an increase in laryngeal cancer (3 incidence cases vs. 0.5 expected).

1.1.2. Styrene-Butadiene Rubber Manufacture (SBR)

A number of studies of workers in the U.S. rubber industry have been reported. While some of these entailed potential exposure to styrene, the majority did not specifically address styrene risk. The following covers those studies that specifically evaluated risks in workers engaged in the manufacture of styrene-butadiene latex rubber.

Meinhardt et al. (1978,1982) conducted a retrospective mortality study at two plants in the U.S. (Texas) following the reporting of two leukemia deaths. A total of 2,756 white male employees who worked a minimum of six months were included (plants A and B combined). Follow-up began in 1943 at one plant, and in 1950 at the other, and ended in 1976. Age-, calendar- and cause-specific rates for U.S. white males were used to calculate expected numbers of death.

Industrial hygiene samples were only available at the time of the study, and indicated time-weighted average exposures to styrene in the range of 0.03 to 12.3 ppm, with means of less than 2 ppm. 1,3-butadiene levels ranged from 0.11 to 174 ppm, with means between 1.24 and 13.5 ppm. Traces of benzene (<1 ppm) were reported in plant A. Results were reported by plant.

In plant A, overall mortality was significantly lower than expected (SMR 80, 95% CI 70-90). There were no elevations in any of the cancer categories analyzed, including respiratory, digestive and urinary organs (lung, pancreas and kidney were not analyzed separately). There were a total of nine deaths due to LH cancers (SMR 155, 95% CI 71-295), 5 of which were leukemias (SMR 202, 95% CI 66-472). Upon closer review of the nine deaths, it was noted that most had started work before the end of 1945 when the hot-temperature batch process with higher exposures to all chemicals was converted to a continuous feed operation. When the analysis was restricted to persons employed between the beginning of 1943 and the end of 1945, all the leukemia cases were found to fall within this time frame (SMR 278, 95% CI 90-648). In plant B, no significant increases were found in any of the causes examined. Total cancer mortality was very low (SMR 53, 95 % CI 26-95). There were only 2 LH cancer deaths observed (vs. 2.6 expected).

Matanoski et al. (1987) reported on an initial cohort study of 13,920 male workers employed one or more years in one of eight styrene-butadiene polymer manufacturing plants (seven in the U.S. and one in Canada). This study was later updated through the end of 1982 (Matanoski et al., 1990) to include a 40-year follow-up. After removing duplicates and correcting other records, the updated analysis covered 12,110 employees who were followed from various times between 1943 and 1982 for a total of 251,431 person-years. Expected numbers of deaths by cause were calculated based on U.S. rates for white and black males (Ontario rates were used for the Canadian plant). Limited quantitative exposure data was available. The average exposure to styrene measured in five of the eight plants ranged between 0.3 and 6.7 ppm (mean 3.5 ppm) for the period 1978-83 (Matanoski, et al., 1993). According to the authors, in addition to styrene and 1,3-butadiene monomer, a large number of chemicals have been used in the SBR manufacturing process over time, including thiocarbamates, diphenylamines, antioxidants, mercaptans, aromatic oils, benzene, hydroquinones and carbon black. Work histories (job titles) were used to assign workers into job categories for analysis; however, no attempt was made to group the jobs according to predicted exposure.

Overall, mortality was significantly lower than expected (SMR 81, 95% CI 78-85). The SMR for all cancers was 85 (95% CI 78-93). No significant excess for cancer of any site was observed. This included respiratory cancer (SMR 84, 95% CI 72-98), pancreatic cancer (SMR 83, 95% CI 54-120), kidney cancer (SMR 103, 95% CI 58-169), and LH cancer (SMR 97, 95% CI 73-126). Breast cancer was not analyzed. A further examination of deaths within the subset of workers involved in production revealed an excess in LH cancers among black employees (SMR 507, 95% CI 187-1107). This was driven primarily by an increase in leukemia (SMR 656, 95% CI 135-1906). The only statistically significant increase across all production workers was in "other" lymphatic cancers (SMR 260, 95% CI 119-494). No significantly increased cancer risks were observed among maintenance or utility workers, although the authors noted that risks for certain digestive system cancers (namely esophagus, stomach and large intestine) were elevated among maintenance workers.

A nested case-control study of 59 LH cancer deaths matched to 193 controls in the cohort study by Matanoski et al. (1990) was reported by Santos-Burgoa et al. (1992). Controls were matched to cases by age, year of employment, duration of employment, plant, and survival of the case. Jobs worked by the cases and controls were assigned estimated exposures (low to high) to styrene, 1,3-butadiene, and other chemicals by a panel of experts. Ranked exposures to styrene and 1,3-butadiene were given a score of 1-10, and an exposure index was calculated based on the number of months spent in a particular job.

Of the 59 LH cancers, 8 were lymphosarcomas, 8 Hodgkin's disease, 26 leukemias, and 17 other lymphatic cancers. Based on the exposure indices, the leukemia cases had considerably higher scores for 1,3-butadiene and styrene than the controls. Matched pair analysis showed a strong association between leukemia and 1,3-butadiene exposure (OR 9.4, 95% CI 2.1-22.9) and a non-significant association with styrene (OR 3.1, 95% CI 0.8-11.2). When exposures to both styrene and 1,3-butadiene were included in a conditional logistic regression model, the odds ratio for 1,3-butadiene remained high (OR 7.4, 95% CI 1.3-41), but the estimated association between leukemia and styrene decreased (OR 1.1, 95% CI 0.23-5.0). Subsequent criticisms concerning the use of controls matched for duration of work prompted the authors to re-analyze their data based on the selection of new controls (Matanoski et al., 1993). The re-analysis showed that the relationship between LH cancer and styrene was not significant, whereas the association with exposure to 1,3-butadiene remained statistically significant.

Delzell et al. (1996) combined the two SBR plants studied by Meinhardt, et al. (1982) and seven of the eight plants studied by Matanoski et al. (1990), re-configured the cohorts, updated mortality records, and re-evaluated the mortality outcomes through the end of 1991. The final cohort consisted of 15,649 males (386,172 person-years) who were followed for an average of 25 years between 1943 and 1991. Complete work histories were available for about 97% of the cohort. A total of 308 work areas were identified from which five process groups and seven subgroups were derived. Age-, race-, gender-, cause-specific U.S. and Canadian rates were used to calculate expected numbers of death. SMRs were computed for all causes and all cancers combined, and for specific types of LH cancers, and were further analyzed by pay classification, year of hire, year of death, years since hire, years worked, and process group. No increase in total LH cancers was noted. However, the authors found an increased risk of leukemia overall (SMR 131, 95% CI 97-174) and among hourly workers (SMR 143, 95% CI 104-191). This was concentrated among subjects dying at ages under 65 years and among those workers with at least 10 years of employment and 20 years of follow-up since first hire (SMR 224, 95% CI 149-323). Leukemia was also increased among workers in polymerization (SMR 251, 95% CI 140-414), maintenance labor (SMR 265, 95% CI 141-453) and laboratories (SMR 431, 95% CI 207-793); all areas with high potential exposure to both styrene and 1,3-butadiene. No other LH cancer subtypes were significantly elevated. The authors concluded that the most likely cause of the leukemia excess was 1,3-butadiene, or a combination of 1,3-butadiene and styrene.

Macaluso et al. (1996) performed a more detailed exposure-based analysis of the same cohort (Delzell et al., 1996) in order to examine leukemia risk in relation to cumulative exposure to 1,3-butadiene, styrene, and benzene. Quantitative estimates of exposure were derived by work area, taking historical changes in processes and tasks into consideration. Time-weighted average (TWA) exposure estimates for styrene ranged from 0-7.7 ppm, with the median averaging

between 0.5-1.1 ppm in work areas entailing any styrene exposure. Mathematical models were used to calculate job- and time-specific average exposures, which were then linked to work histories to obtain cumulative exposure estimates by individual. These were analyzed using SMRs (based on general population rates), stratified internal comparisons (rate ratios), and Poisson regression analyses. The excess leukemia was more strongly and more consistently associated with 1,3-butadiene exposure than with styrene exposure. In particular, a positive dose-response relationship between cumulative butadiene exposure (ppm-years) and leukemia was observed after controlling for styrene exposure. The same was not true for styrene after controlling for butadiene exposure.

Sathiakumar et al. (1998) performed a further analysis of the Delzell et al. (1996) cohort to assess the association between 1,3-butadiene and styrene exposure and cancers of the lung and gastrointestinal system (in addition to LH cancers). U.S. and Ontario (Canada) general population mortality rates were used to compute SMRs covering the period 1943-91. The overall SMR for all cancers was 93 (95% CI 87-99). The only significantly increased cancer SMR was that for leukemia among hourly employees (SMR 143, 95% CI 104-191). When the analysis was restricted to employees with 10 or more years of employment and 20 or more years since hire, the risk increased (SMR 224, 95% CI 149-323), with the highest risk among black employees. Lung, pancreatic and kidney cancer SMRs were all at or below 100.

Using revised exposure assessments of 5 of the 7 facilities in the original study, Delzell and coworkers (2001) evaluated leukemia risk among 13,130 men employed in the SBR industry from 1943-1991. In analyses that assumed workers had only been exposed to styrene, there was a positive association with risk for leukemia; however, after adjusting for butadiene and dimethyldithiocarbamate exposure, there was no positive association of styrene exposure and leukemia.

1.1.3. Reinforced Plastics and Composites (RPC) Industry

Mortality has been studied in three cohorts of RPC workers: Ruder et al. (2004) (update of Okun et al., 1985); Wong et al., (1994) (update of Wong, 1990); and Kogevinas et al., (1993, 1994). Kogevinas et al. (1993, 1994) includes updates of cohorts previously published by Coggon et al. (1987) and Harkonen et al. (1984). Cancer incidence, rather than mortality, has been studied in one cohort Kolstad, et al. (1994, 1995), part of which was included in the Kogevinas et al. (1993, 1994) cohort.

Okun et al. (1985) studied a cohort of 5,021 boat-builders, 2,060 of whom were classified as having high exposure to styrene. A total of 176 deaths were observed compared to 195.3 expected based on the general US population (SMR = 90). Workers were divided into high and minimal exposure groups based on industrial hygiene surveys, but no TWA exposures or other levels were presented. Among the high exposure group, 47 deaths were observed vs. 41.5 expected (SMR = 113). No leukemia or lymphoma deaths were observed. There was one death each from cancers of the respiratory and urinary systems. Among workers with minimal exposure to styrene, there was a non-significant increase in respiratory cancer death (SMR 155, 95 % CI 87-255). An update of this study (Ruder et al., 2004) provided an average of 26 years of follow-up for 5,201 reinforced plastics workers. There was no increase in overall lymphatic and

hematopoietic cancers, nor in subcategories of leukemia, Hodgkin's disease, lymphoma in the overall cohort, or among high or low exposure subcohorts. SMRs in the high exposure subcohort were not greater than in the low-exposure subcohort.

Wong (1990) reported on an initial study of 15,826 workers in 30 plants employed for at least 6 months in the RPC industry in the U.S. during 1948-1977. Mortality patterns were subsequently updated through 1989 (Wong et al., 1994), with an average follow-up of 19.6 years (307,932 total person years). SMRs were calculated using age, sex, race and calendar-specific U.S. death rates. Historical occupational hygiene measurements (i.e., time-weighted average exposures by job and time period) for styrene were used to develop a job-exposure matrix for the purpose of estimating personal exposure indices for each member of the cohort, including duration of exposure and cumulative exposure (ppm-years). A personal cumulative exposure was constructed for each individual based on estimated time weighted average exposure by job title, by year, and the years in each job. However, the authors noted that exposures may have been underestimated for several reasons: (1) the exposure estimates were conservative; (2) exposure calculation was truncated at 1977; (3) 3% of the population had no job titles and were assigned an exposure index of 0; and (4) a small number of jobs were non-specific in nature and were assigned a value of 5 ppm.

Overall, there were significant increases in mortality from all causes (SMR 108, 95% CI 103-113), all cancers combined (SMR 116, 95% CI 105-127), esophageal cancer (SMR 198, 95% CI 105-322), lung cancer (SMR 141, 95% CI 120-164), cancer of the cervix uteri (SMR 284, 95% CI 136-521), and cancer of other female genital organs (SMR 202, 95% CI 107-345). Non-significant increases were observed in kidney cancer (SMR 175, 95% CI 98-289) and pancreatic cancer (SMR 113, 95% CI 68-177), while breast cancer mortality was low (SMR 62, 95% CI 34-108). Most of the mortality increases were among workers who were employed for only 6 months to one year or who had very low cumulative exposure (<10 ppm-years). There was no increased mortality from LH cancers overall or from any specific sub-category, including leukemia. Nor was there any relationship between several indices of styrene exposure (i.e., time since first exposure, duration of exposure, average exposure, cumulative exposure, or job category) and any type of LH cancer. Two Cox regression models were run; one using only cumulative exposure as a variable and the other with both cumulative exposure and duration of exposure as variables. Neither analysis showed styrene exposure to be a significant risk factor. Thus, based on analysis of SMRs and Cox regression models, LH cancer deaths were not increased in relation to styrene exposure. Increased lung cancer deaths were considered to be smoking related, based on a prior nested case-control study (Wong, 1990). Similarly, an increase in kidney cancer among workers with 10 or more years of employment and greater than 100 ppm-years of cumulative exposure did not show any relation to styrene exposure when analyzed using the Cox proportional hazards model. Because there was no upward trend in any of these causes of death in relation to styrene exposure based on duration of employment, duration of exposure, cumulative exposure, or job category, the authors concluded that the increased mortality was not likely related to exposure to styrene. Instead, they suggested that these effects were likely related to low socioeconomic status, smoking, and/or lifestyle factors characteristic of short-term workers.

Kogevinas et al. (1994) reported on 40,688 workers employed in the European RPC industry (no minimum employment period). This study was a combination of eight sub-cohorts from six European countries (Denmark, Finland, Italy, Norway, Sweden and the United Kingdom), and included the cohorts reported by Coggon, et al. (1987) and Kolstad et al. (1994, 1995). Each cohort had a different follow-up period ranging from 1945-1990. Overall, there was an average follow-up of 13 years (539,479 person years). About 60% of the cohort worked less than two years. Within the Danish sub-cohort, which represented 39% of the total cohort, over 80% worked less than two years. Mortality rates, by country, derived from the WHO mortality data bank, were used for comparison. Exposure data was derived from about 16,500 personal air samples collected between 1950 to 1990, and 18,500 measurements of styrene metabolites in urine collected during the late 1980s. The data from Denmark was considered representative of all countries and showed levels of about 200 ppm in the 1950s, 100 ppm in the late 1960s, and 20 ppm in the late 1980s. According to the authors, exposure levels decreased considerably during the study period in all six countries. Detailed analyses were performed by job category, duration of exposure to styrene, average level (intensity) of exposure, and cumulative exposure (ppm-years). Poisson regression was used for internal comparisons. Review of the full IARC Report reveals that exposures in some of the other countries were considerably higher than in Denmark; thus internal comparisons may have been unreliable.

Among the total cohort, there was no increase in deaths from all causes (SMR 92, 95% CI 88-95) or all cancers combined (SMR 87, 95% CI 81-94). LH cancers likewise were not elevated overall (SMR 93, 95% CI 71-120), nor among exposed workers (SMR 96, 95% CI 71-127). There was no association with either duration of exposure or cumulative exposure. LH cancer deaths, however, showed increases with time since first exposure and average styrene exposure based on a Poisson regression model. In this study, a cumulative exposure was constructed for each member based on available job descriptions, industrial hygiene measurements, and years worked. Average Exposure was calculated for each person by dividing the cumulative exposure by the duration of exposure; thus, it seems illogical that the dividend (average exposure) is related to tumors if neither the numerator (cumulative exposure) nor the denominator (duration of exposure) were related to tumor occurrence. No increases in breast or lung cancer deaths were observed, nor was there any relationship to cumulative exposure. In addition, no significant excess risks were observed for either kidney or pancreatic cancer among the exposed workers overall. However, mortality from kidney cancer increased with cumulative exposure, but decreased with time since first exposure. Mortality from pancreatic cancer increased with cumulative exposure, and was highest among workers with 20 or more years since first exposure to styrene.

Kolstad et al. (1994) evaluated LH cancer incidence, rather than mortality, in a cohort of 54,000 workers within 368 companies in the Danish RPC industry. Approximately 13,000 were members of the cohort studied by Kogevinas et al. (1994). Sixty-one percent of the cohort had less than 1-year employment. The number of exposed workers over the study time period was estimated at 36,525 workers according to dealers, compared to 28,518 according to the company owners. The overlap agreed to by both sources was 26,784, but the dealers' estimate was used in the analysis. Thus, the total number of workers exposed to styrene was in question. The average annual styrene levels measured at 128 of the companies were used to represent exposures over the study period, and ranged from 180 ppm in 1964-70 to 43 ppm in 1976-88. No individual

exposure data were available. National cancer incidence rates standardized for age, gender and year of diagnosis were used to calculate SIRs. Internal comparisons were made using Poisson regression models. Detailed analyses were performed by year of first employment, duration of employment (<1 year vs > 1 year), and years since first employment.

Among all companies producing reinforced plastics, there was a non-significant increase in the incidence of LH cancer (SIR 120, 95% CI 98-144). In workers with > 10 years since first employment, leukemia incidence was significantly increased (SIR 157, 95% CI 107-222). This was confined to workers first employed between 1964-1970 (SIR 170, 95% CI 109-249), and those who worked less than one year (SIR 234, 95% CI 143-361). Among workers with less than 10 years since first employment, the only significant increase was in Non-Hodgkin's lymphoma (SIR 168, 95% CI 103-253). There are a number of issues that make interpretation of this study difficult: First, by the authors' own admission the exposure status was not determined for any individual in the cohort; i.e., no attempt was made to determine if individuals were actually in jobs where they were exposed to styrene. Secondly, the authors estimated that 43% of the employees of the companies were actually involved in reinforced plastics operations; no attempt was made to determine what proportion of that 43% was involved in high exposure jobs, such as lamination. Thirdly, which companies to include as being involved in reinforced plastics was based on recall by two resin dealers. The authors concluded that they had a better recall than owners of the companies because they found a threefold excess risk for non-Hodgkin's lymphoma and leukemia in 1,774 employees of companies producing reinforced plastics when analyzed according to the dealers' assessment, but not according to the employers'. Therefore, they only presented analysis based on the dealers' assessments. Fourthly, from a sample of 671 employees in 8 companies, the estimates of duration of exposure from the pension fund were underestimated for 40% and overestimated for 13% (as far as whether they had worked less or more than 1 year). Fifthly, 28 leukemias were recorded for companies where it was estimated that between 1 and 49% of the workforce was involved in reinforced plastics (23,688 employees). In companies where it was estimated that more than 50% of the workers were involved in reinforced plastics (12,837 workers), 14 cases of leukemia were reported. No attempt was made to assess whether any of these 42 employees were actually involved in reinforced plastics or were laminators.

Kolstad et al. (1995) reported on a second study examining the mortality and incidence of solid cancers in the same cohort of Danish RPC workers, using the same inclusion criteria. While overall mortality was increased by about 10% (SMR 110, 95% CI 106-114), the authors point out that a somewhat larger excess mortality was found among the workers in companies with no exposure to styrene (SMR 117, 95% CI 111-123). The same was generally true for the incidence of solid tumors. Among all the site-specific cancers studied, only pancreatic cancer showed increased risk among workers in the RPC industry compared with workers not exposed to styrene (IRR 2.2, 95% CI 1.1-4.5).

An additional study evaluated cancer risks in relation to styrene exposure among 2,580 subjects in Finland (Anttila et al., 1998). Exposures were relatively low (average urinary mandelic acid 2.3 mmol/L). There was no increase in the incidence of any cancer, including lymphatic and hematopoietic cancers.

1.1.4. Summary of Epidemiologic Data

In both the styrene/polystyrene (PS) and styrene-butadiene rubber (SBR) manufacturing segments, exposures to styrene were generally very low, and confounded by exposures to a number of other chemicals, many of which are known or suspected carcinogens. Furthermore, the cancer increases are inconsistent and of mixed types, and often are not among workers with the highest exposure to styrene. Recent studies among SBR workers have shown the increase in leukemia to be most strongly associated with 1,3-butadiene and dithiodicarbamate exposure. Studies in the reinforced plastics (RPC) industry typically involved considerably higher levels of styrene and less potential for confounding, but included many short term workers, and were further hampered by both the lack of individual exposure data and the potential misclassification of exposed workers (primarily in the large Danish cohort).

None of the studies showed any significantly increased risks or suggested any relationship between styrene and cancer of the kidney. Similarly, no studies reported increased breast (mammary) cancer; however, most of the studies did not include female workers due to small numbers. All but one of the studies showed no increased risk from lung cancer. Only the study by Wong (1990) identified a significant increase in lung cancer, but a subsequent nested case-control study found no relationship to styrene exposure. A non-significant increase in lung cancer among SBR maintenance workers (Sathiakumar et al., 1998) was concluded to be most likely caused by smoking or some other unidentified occupational exposure.

Pancreatic cancer was increased in both the Kogevinas et al. (1994) and Kolstad et al. (1995) studies, but only significantly in the latter. Both showed a relationship with duration of employment and cumulative exposure. However, these results must be tempered by the fact that the Kolstad cohort of Danish RPC workers represents the largest subgroup within the Kogevinas study. Therefore, these cannot be viewed as independent and corroborating findings. The study by Wong et al. (1994) found a small, non-significant increase in pancreatic cancer, which was concentrated among employees working less than one year. No other studies reported an increase in pancreatic cancer. Thus, based on an increase in the Danish cohort of RPC workers alone, it is difficult to assign much weight to this finding. Additional supporting evidence is needed to permit a conclusion on the potential relationship between styrene exposure and pancreatic cancer.

Increases in LH cancer were observed in all three industry segments, but not consistently. As discussed, studies in the styrene monomer, styrenic polymers and SBR industries cannot be given much weight due to the strong role of confounding exposures, particularly in the SBR industry. Furthermore, not all studies reported breakdowns by type of leukemia/lymphoma, although where they did, inconsistencies of the type associated with styrene exposure were reported. In addition, risks were not highest in workers with the greatest potential exposure. Exposures to styrene have typically been highest in the RPC industry where there is also less confounding by known or suspected carcinogens. Therefore, studies in this industry segment provide a better basis for assessing styrene's carcinogenicity. Studies of European RPC workers, where measured exposures were the highest, showed some results that were suggestive of an increase in LH cancer risk; one being increased risk with average intensity of exposure and time since first exposure; the other being highest risk in workers employed at times of highest

estimated exposure. In contrast, however, risk did not increase with increasing cumulative exposure to styrene, and occurred primarily in short-term workers. There was no increased risk in LH cancers among workers in the U.S. RPC industry, nor among all cohorts of RPC workers combined. Thus, on the basis of all the available human data on styrene, the weight of evidence continues to support the conclusion that styrene presents no demonstrable risk of cancer in humans, either in LH or pancreatic cancers, or in sites suggested by animal models.

1.2. Long-term Animal Studies

Styrene has been evaluated in eight rat and five mouse long-term carcinogenicity studies. These studies encompassed several routes of exposure and strains of rodents. Inhalation studies, the most likely route of human exposure due to the high vapor pressure of styrene, have been conducted using rats (Cruzan et al., 1998; Conti et al., 1988; and Jersey et al., 1978) and CD-1 mice (Cruzan, 2001). Oral gavage studies have been reported for rats (Conti et al., 1988; National Cancer Institute, 1979a; and Ponomarkov and Tomatis, 1978) and for mice (NCI, 1979a; and Ponomarkov and Tomatis, 1978). Oral studies of a mixture of 70% styrene and 30% β-nitrostyrene have also been conducted in rats and mice (NCI, 1979b). Styrene given in the drinking water has been evaluated in rats (Beliles et al., 1985).

1.2.1. Carcinogenicity Studies in Rats

In the following studies, no styrene-related increased tumor incidences were reported (see (appendix B for details):

a.   Cruzan et al. (1998) exposed Sprague-Dawley (SD) rats by inhalation at 50, 200, 500 and 1,000 ppm styrene, 6 hrs/day, 5 days/week for 104 weeks.

b.   Jersey et al. (1978) exposed SD rats by inhalation at 600 and 1000 ppm styrene (males exposed 78 weeks, females 87 weeks) 6 hrs/day, 5 days/week and terminated the study at 104 weeks.

c.   Conti et al. (1988) administered 50 and 250 mg/kg/day styrene by gavage 4 days/week to SD rats for 12 months and observed until death.

d.   Styrene was administered to F344 rats at 500 mg/kg/day for 103 weeks and at 1,000 and 2,000 mg/kg/day 5 days/week for 78 weeks and terminated at 105 weeks (NCI, 1979a).

e.   Styrene was administered to pregnant (gestation day 17) BDIV rats at 1350 mg/kg and to their offspring at 500 mg/kg once per week for 120 weeks (Ponomarkov and Tomatis, 1978).

f.   A mixture of 70% styrene and 30% β-nitrostyrene was administered 3 days/week to F344 rats at styrene doses of 350 and 700 mg/kg/day to males and 175 and 350 mg/kg/day to females for 79 weeks and terminated at 108 weeks (NCI, 1979b).

g.   Styrene was given in the drinking water to SD rats at 125 and 250 ppm for 104 weeks (Beliles et al., 1985).

Based on this review of the chronic rat studies on styrene, only two potential tumor sites have been identified for evaluation:

- increased mammary tumors in females (Conti et al.; 1988) and
- increased testicular tumors in males (Cruzan et al., 1998).

1.2.1.1. Mammary Tumors in Female Sprague-Dawley Rats

Maltoni's laboratory conducted chronic studies on styrene both by inhalation and by gavage (Conti et al., 1988). They reported the gavage study as negative, but reported increased mammary tumors, both total and malignant, in female SD rats exposed to styrene by inhalation 4 hrs/day 5 days/week for 12 months (and then held until death). Very few data are presented in the report; in particular, mortality experience is not presented. Malignant mammary tumors were diagnosed for 6/60 (10%), 6/30 (20%), 4/30 (13%), 9/30 (30%), 12/30 (40%) and 9/30 (30%) for rats inhaling 0, 25, 50, 100, 200 or 300 ppm, respectively. For total mammary tumors, the rates were 34/60 (57%), 24/30 (80%), 21/30 (70%), 23/30 (77%), 24/30 (80%) and 25/30 (83%) for the respective exposure levels. The authors stated that the increases were statistically significant but the analyses were not presented. Although the frequency of spontaneously developing mammary tumors is high in female SD rats (van Zwieten et al., 1994), historical control data for the authors' laboratory were not given in the paper. However, Maltoni (1978) had previously written "It should be pointed out that the incidence of mammary tumors is quite high in the colony of rats we employed, that some fluctuation of that incidence is currently observed from group to group, and that the results of the other experiments do not support a correlation between styrene exposure and mammary tumors."

In a recent rat study [performed according to current Good Laboratory Practices (GLPs) and study design criteria] (Cruzan et al., 1998), groups of 60 male and 60 female rats were exposed to 0, 50, 200, 500, or 1,000 ppm styrene for 6 hr/day, five days/week for 104 weeks. Factors implemented in the study design and conduct included: sentinel animals to monitor spontaneous infectious disease; study designed to EPA and OECD guidelines; large group sizes, interim necropsies (10 additional rats/sex/group) and clinical pathology evaluations throughout the study; characterization of test article and chamber atmospheres; histopathologic examination; GLP compliance and external GLP monitor; and external peer review of pathology.

In this study there were no increased tumors related to styrene exposure. On the contrary, Cruzan et al. (1998) reported a dose-dependent decrease in mammary tumors in females. Mammary adenocarcinomas were diagnosed in 20/61 (33%), 13/60 (22%), 9/60 (15%), 5/60 (8%) and 2/60 (3%) female rats exposed to 0, 50, 200, 500 or 1,000 ppm styrene, respectively, for two years. A less dramatic decrease in benign mammary fibroadenomas (including those with epithelial atypia) was seen, with the incidence being 27/61 (44%), 22/60 (37%), 18/60 (30%), 21/60 (35%) and 19/60 (32%) for the above exposure levels, respectively. In this study, females exposed to 500 and 1,000 ppm weighed equally less than controls throughout the study (~25% at

termination). However, there was a dose-related increase in survival; at termination, survival was 48, 47, 48, 67 and 82% in females exposed to 0, 50, 200, 500 and 1000 ppm, respectively.

Jersey et al. (1978) reported no styrene-related increases in mammary tumors in SD rats at 600 and 1,000 ppm. Mammary gland adenocarcinomas were increased in females exposed to 600 ppm styrene, but not in females exposed to 1,000 ppm. The authors reported 7/85 (8%) rats exposed to 600 ppm styrene with mammary adenocarcinoma compared to 1/85 (1%) control rats and 0/85 (0%) rats exposed to 1,000 ppm styrene. The authors considered mammary adenocarcinomas not to be related to styrene exposure due to the lack of dose response and the fact that the concurrent controls had a lower incidence of mammary adenocarcinomas than historical controls. The authors presented historical control data from eight studies conducted in the same laboratory over the five years prior to the styrene study in which the incidence of mammary adenocarcinomas averaged 5.8% and ranged from 0 to 9%. They found no association between styrene inhalation and the incidence of mammary fibroadenomas, the common benign mammary tumor of rats; the incidences found were 60/85 (71%), 64/85 (75%) and 63/85 (74%) for rats exposed to 0, 600 ppm and 1,000 ppm styrene, respectively.

Conti et al. (1988), in a gavage study using SD rats, reported incidences of malignant mammary tumors in females of 12, 30 and 12% for daily doses of 0, 50, and 250 mg/kg/day. Beliles et al. (1985), who administered styrene in the drinking water at 0, 125 and 250 ppm (12 and 21 mg/kg/day) for two years, reported total mammary tumors in female SD rats as 56, 67, and 75% respectively. They did not separately report malignant mammary tumors. In gavage studies in F344 rats, which have a much lower incidence of mammary tumors than SD rats, no increase in mammary tumors was found when styrene was administered alone or with β-nitrostyrene. When styrene was administered by gavage using daily doses of 0, 500, 1,000, or 2,000 mg/kg/day, one of 50 low dose females had a malignant mammary tumor; no other females in the study had malignant mammary tumors. The incidences of benign mammary tumors in females were 5/40 (12.5%), 2/50 (10%), 3/50 (15%) and 0/50 (0%) for females treated with 0, 500, 1,000, or 2,000 mg/kg/day styrene (NCI, 1979a). When a mixture of 70% styrene and 30% β-nitrostyrene was administered at styrene daily doses of 0, 175 and 350 mg/kg/day, no malignant mammary tumors were found in any female in the study. The incidences of benign mammary tumors were 2/20 (10%), 5/50 (10%) and 7/50 (14%) in females treated with 0, 175 and 350 mg/kg/day styrene (NCI, 1979b). Ponomarkov and Tomatis (1978) reported total mammary tumor incidences of 49 and 16% in female BDIV rats whose mothers were treated with 0 or 1,350 mg/kg on day 17 of gestation with the offspring subsequently treated with 0 or 500 mg/kg styrene once/week for 120 weeks.

1.2.1.2. Testicular Tumors in Male Sprague-Dawley rats

Cruzan et al. (1998) reported a significant trend for increased benign testicular interstitial cell tumors in male Sprague-Dawley rats. The incidences were 2 (3.3%), 2 (3.3%), 2 (3.3%), 4 (7.4%), 6 (11.5%) in males exposed to 0, 50, 200, 500 and 1,000 ppm, respectively. These tumors were judged not treatment-related because the values for all groups were within the historical control range for the laboratory (up to 13%); none of the incidences was statistically different from control by pairwise comparison; and there was no increase in pathology normally associated with chemically-induced testicular tumors; i.e., there was no increase in the incidence

or degree of interstitial cell hyperplasia or tubular atrophy. The authors concluded these testicular tumors were unrelated to the inhalation of styrene.

Conti et al. (1988) did not report incidence of testicular tumors, leading to the conclusion that the incidence was not increased in either the gavage or inhalation study of SD rats. Beliles et al. (1985) reported incidences of 14, 4 and 4% for male SD rats given styrene in the drinking water at 0, 125 and 250 ppm, while Jersey et al. (1978) reported incidences of 6, 0, and 0% for male SD rats exposed to 0, 600 and 1,000 ppm, respectively. In the NCI studies using F344 rats, which have extremely high incidence of interstitial cell tumors, the incidences of interstitial cell testicular tumors were: 85, 89, and 96% for doses of 0, 500, and 1,000 mg/kg/day styrene and 79, 81 and 85% for doses of 0, 350 and 700 mg/kg/day styrene as a mixture with β-nitrostyrene. Ponomarkov and Tomatis (1978) reported no testicular tumors in male BDIV rats exposed to 0 or 500 mg/kg styrene once per week.

1.2.1.3. Analysis of Tumors in Rats

Increased mammary tumors were reported in only one (Conti et al., 1988) of eight studies; similar or greater doses in the other studies did not result in increased mammary tumors. A dose-related decrease in mammary tumors was reported in the most recent study covering the range of doses used by Conti and coworkers and extending to 5-fold greater daily doses (Cruzan et al., 1998) and up to 10-fold greater lifetime dose. When the mammary tumor data from the eight rat studies are compared based on the total lifetime administered dose (from 1.9 g/kg to 792 g/kg), there is no dose-response relationship to styrene treatment. Based on the weight of the evidence, it is concluded that exposure to styrene does not result in increased mammary tumors in female rats.

Mammary Tumor Results in Female Rats Treated with Styrene							
Strain	Route of Exposure	Administered Dose	Daily[a] Dose (mg/kg/day)	Exposure (months)	Lifetime Dose (g/kg)	Reported[a] Result	Reference
SD	Inhalation	25 ppm	7.3	12	1.9	i	Conti et al., 1988
SD	Inhalation	50 ppm	14.7	12	3.9	i	Conti et al., 1988
SD	Inhalation	100 ppm	29	12	7.7	i	Conti et al., 1988
SD	Water	125 ppm	12	24	9.9	=	Beliles et al., 1985
SD	Inhalation	50 ppm	22	24	11.6	=	SIRC, 1996
SD	Gavage	50mg/kg/d	50	12	13.2	=	Conti et al., 1988
SD	Water	250 ppm	21	24	14.9	=	Beliles et al., 1985
SD	Inhalation	200 ppm	58	12	15.3	i	Conti et al., 1988
SD	Inhalation	300 ppm	87	12	23	i	Conti et al., 1988
F344	Gavage (m)	175 mg/kg/3x	105	18	42	=	NCI, 1979 (b)
SD	Inhalation	200 ppm	58	24	45	=	SIRC, 1996
BDIV	gavage	500 mg/kg/wk	100	24	53	=	Ponomarkov, Tomatis, 1978
SD	Gavage	250 mg/kg/d	250	12	66	=	Conti et al., 1988
F344	Gavage (m)	350 mg/kg/3x	210	18	84	=	NCI, 1979 (b)
SD	Inhalation	500 ppm	218	24	115	d	SIRC, 1996
SD	Inhalation	600 ppm	262	20	115	=	Jersey et al., 1978

					Lifetime		
Strain	Route of Exposure	Administered Dose	Daily[a] Dose (mg/kg/day)	Exposure (months)	Dose (g/kg)	Reported[a] Result	Reference
SD	Inhalation	1000 ppm	436	20	192	=	Jersey et al., 1978
SD	Inhalation	1000 ppm	436	24	230	d	SIRC, 1996
F344	Gavage	500 mg/kg/d	500	24	264	=	NCI, 1979 (a)
F344	Gavage	1000 mg/kg/d	1000	18	396	=	NCI, 1979 (a)
F344	Gavage	2000 mg/kg/d	2000	18	792	=	NCI, 1979 (a)

Mammary Tumor Results in Female Rats Treated with Styrene (continued)

[a] Based on total female rats with benign or malignant tumor.
i - reported increase,
= - no difference from control,
d - reported decrease,
(m) - mixture 70% styrene, 30% β-nitrostyrene, dosed 3 times per week.

A positive trend in benign testicular interstitial cell tumors was found in one study (Cruzan et al., 1998) although, for the reasons stated above, the authors did not attribute this to styrene exposure. An increase in testicular tumors was not reported in any of the other 8 rat studies. Therefore, it is concluded that styrene exposure does not result in increased testicular tumors in rats.

Overall, the eight chronic studies in rats exposed to styrene by inhalation or oral routes provide convincing evidence that styrene is not carcinogenic in rats.

1.2.2. Carcinogenicity Studies in Mice

Five chronic studies have been conducted in mice, one by inhalation and four by gavage. The only tumor type that has been increased in styrene-exposed mice is lung tumors. No increases in other tumor types have been reported. The individual long-term studies are described in Appendix B. The data relevant to mouse lung tumors are presented below.

A two-year inhalation study using CD-1 mice has recently been completed (Cruzan et al., 2001). Design considerations were similar to those of the Cruzan et al. (1998) rat study described previously. Groups of 50 male and 50 female mice were exposed to 0, 20, 40, 80 or 160 ppm six hrs/day, five days/week for 98 weeks for females and for 104 weeks for males. Due to elevated mortality in control females (23 of 50 mice), the surviving females were terminated six weeks earlier than originally scheduled; all four treated groups had greater survival. Interim terminations of an additional 10 males and 10 females from each exposure level occurred after 52 and 78 weeks. Male and female mice exposed to 80 and 160 ppm had decreased body weights over the course of the study. Increased incidences of bronchiolo-alveolar adenomas were seen in males exposed to 40, 80 or 160 ppm for 24 months with no dose-response, but the incidences of bronchiolo-alveolar carcinomas were not increased. In females, the incidences of bronchiolo-alveolar adenomas in the groups exposed to 20, 40, and 160 ppm, but not 80 ppm, for 22.5 months were increased. In addition, females exposed to 160 ppm had an increased incidence (14%) of bronchiolo-alveolar carcinomas although the incidence barely exceeded the historical

control range. The historical control range was 0-4% for the laboratory (5 oral studies) and 0-13.5% for Charles River (9 oral studies).

No increase in lung tumors was seen in males or females after 12 or 18 months. The increase seen after 24 months was largely in small tumors, as demonstrated by a decreased average tumor size, especially obvious in males, again emphasizing that these were late-developing tumors. No difference in tumor morphology (solid vs. papillary, visual appearance of cells, immunostaining for markers of Clara and type II cells) between control and treated mice was seen. Non-neoplastic histopathologic lesions of the terminal bronchioles were seen at all exposure concentrations in a dose-responsive pattern at all interim and the terminal necropsies. These lesions included decreased eosinophilic staining of Clara cells, cellular crowding, and epithelial hyperplasia extending into alveolar ducts.

Ponomarkov and Tomatis (1978) reported an increased incidence of pulmonary tumors in O20 mice, a strain obviously very susceptible to the formation of lung tumors. When the data of the untreated and olive oil treated control mice were combined, 57% of the males and 57% of the females developed lung tumors. The study design was unconventional, with pregnant females gavaged with 1,350 mg/kg body weight on day 17 of gestation. Pups were then given a very high dose of 1,350 mg/kg by gavage weekly following weaning. Dosing was terminated after 16 weekly doses due to toxicity. All mice were held until spontaneous death, euthanasia due to moribund condition, or 120 weeks. This dose level clearly exceeded the maximum tolerated dose with higher preweaning mortality in the styrene treated mice than controls treated with olive oil vehicle (43% versus 22%) and 50% mortality in males and 20% in females by the twentieth week post-weaning (male olive oil controls had 5% mortality and females had 0% mortality at twenty weeks). The authors reported lung neoplasms in 20/23 (87%) of males and 32/32 (100%) of females given styrene compared to 8/19 (42%) of males and 14/21 (67%) of females receiving olive oil gavage, after adjusting for early mortality. Lung tumors were found in 34/53 (64%) untreated control males and 25/47 (53%) untreated control females. The incidence in the styrene treated male mice was significantly higher than the olive oil controls only, while in females, the styrene treated group was significantly greater than either control group. In their study conclusions, the authors cautiously note "the increased incidence and early appearance of lung tumors could possibly indicate a carcinogenic effect for styrene in O20 mice. This experiment, however, has severe limitations, since the dose used was obviously very high, causing severe toxic effects and an early mortality. Results from additional studies are needed before a final evaluation of the carcinogenicity of styrene in rodents can be made."

Using a similar study design, no increases in lung tumors occurred in C57 Bl mice when 300 mg/kg body weight (bw) was given to pregnant females on day 17 of gestation and then to the offspring weekly following weaning until 120 weeks. In C57 mice, only 1/27 (4%) of styrene-treated males had lung tumors compared to 3/12 (25%) of the olive oil control males and 5 of 47(11%) untreated controls (Ponomarkov and Tomatis, 1978). In females, the incidences were 1/24, 1/13, and 1/47 for the styrene treated, olive oil controls and untreated controls, respectively.

The National Cancer Institute also conducted a gavage study of styrene bw mice (1979a). In this study, B6C3F1 mice were gavaged with either 150 or 300 mg/kg bodyweight, five days/week for

78 weeks, and were held for an additional 13 weeks prior to termination. There was slightly increased mortality in males but survival was considered adequate for evaluation of late-occurring tumors. Male mice were noted to have an increased incidence of combined pulmonary adenomas and carcinomas. Alveolar/bronchiolar adenomas or carcinomas were noted in 6/45 (13%) low dose and 9/49 (18%) mice given the high dose level. Lung tumors were not noted in any of the 20 concurrent (vehicle) controls, which was unusual since the historical control incidence for untreated controls was 12% (range 0-20%). There were insufficient historical control data for vehicle-treated control mice to establish if their incidence of lung tumors differed from that of untreated historical controls. The response in the mice receiving 150 mg/kg/day was in the middle of the historical control range, while the response of the mice receiving 300 mg/kg/day was at the high end of the historical control range. In females, there were no alveolar/bronchiolar carcinomas in any group and the incidence of adenomas was 0/20, 1/44 (2%) and 3/48 (6%) for female mice treated with 0, 150 or 300 mg/kg/day. The discrepancy in control incidence and response in only one sex caused difficulty in interpretation and the NCI conclusion was that "under the conditions of this bioassay, no convincing evidence for the carcinogenicity of the compound was obtained in Fischer 344 rats or B6C3F1 mice of either sex."

A mixture of 70% styrene: 30% β-nitrostyrene was administered by gavage 3 times per week at styrene doses of 204 and 408 mg/kg/dose (β-nitrostyrene doses were 87.5 and 175 mg/kg/dose) for 78 weeks to B6C3F1 mice, which were held for an additional 14 weeks prior to termination (NCI, 1979b). There was slightly increased mortality in males (14 mice died early in the study) but survival was considered adequate for evaluation of late occurring tumors. The incidence of pulmonary adenomas and carcinomas combined was 0/20, 11/50 (22%), and 2/36 (6%) for control, low dose and high dose male mice, respectively. In females, the incidences of combined adenomas and carcinomas of the lung were 0/10, 2/49 (2%) and 0/46 for groups treated with 0, 204 and 408 mg/kg/day styrene. The NCI concluded there was no evidence of carcinogenicity in this study. Because there was no increase in tumor incidence among the high-dose mice, it was concluded that styrene did not cause increased lung tumors at the low dose. Again, there was a small control group and a historical control range of 0 to 20% in untreated controls.

Styrene did not induce lung tumors in a screening assay in A/J mice, a strain highly susceptible to this neoplasm (Brunnemann et al., 1992). Intraperitoneal injections of 20 μmol styrene (66 mg/kg/dose) three times per week for 7 weeks did not result in increased lung tumors when the mice were sacrificed 20 weeks later. The incidences were: controls 1/25 (4%), styrene 3/25 (12%), positive control 25/25 (100%).

1.2.2.1. Analysis of Tumors in Mice

Five chronic bioassays, one by inhalation and four by gavage, have indicated that there is only one tumor site of concern in mice, the lung.

A two-year inhalation study resulted in increased incidence of late onset lung tumors (detected mostly as small lesions, only at 24 months) and in the presence of lung pathology. Significantly, increased lung tumors were reported in male and female O20 mice at a very toxic dose of styrene, which caused significant early mortality. In a gavage study, there was a significant trend

for increased lung tumors in male B6C3F1 mice, but the incidence for each group was within the historical control range and no effect was seen in female mice. In contrast, increased lung tumors were not found in two other mouse gavage studies (NCI, 1979b; Ponomarkov and Tomatis, 1978) at dose levels comparable to the NCI study of styrene.

1.2.3. Summary of Long-term Animal Carcinogenicity Studies

The numerous animal carcinogenicity studies of styrene indicate that it is not a carcinogen in rats. This conclusion is based upon a review of all studies and encompasses both inhalation and oral routes of exposure along with subcutaneous and intraperitoneal injection dosing. Although neoplasms were statistically identified as related to styrene exposure in a few of the studies, the results were not repeatable by other investigators nor were the tumors related to dose level. Given the numerous opportunities for styrene to have demonstrated a carcinogenic effect, the lack of any consistent trend is noteworthy.

On balance, the available studies indicate that styrene causes increased lung tumors in mice. Although four studies by gavage administration had inconsistent results and were flawed by design deficiencies, lung was identified as a possible target organ. A two-year inhalation study conducted according to state-of-the-art methodology resulted in an increase of late-onset lung tumors (detected as small lesions, only at 24 months) and in the presence of lung pathology.

2. Mode of Action Data

2.1.Target organs in Laboratory Animals

2.1.1. Lung

Inhalation or oral exposure to styrene has been reported to produce lung toxicity in mice, but not in rats. Effects in mice have been seen consistently in the terminal bronchioles, but no effects are reported in alveolar cells.

Gadberry et al. (1996) and Carlson (1997a) reported increased levels of the enzymes gamma-glutamyl transpeptidase (GGT) and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid (BALF) obtained at necropsy 24 hours after the intraperitoneal (ip) injection of 600 mg/kg or greater styrene in mice. Histopathologic evaluation of the lungs did not demonstrate which cells were damaged. Ip injection of styrene oxide (SO, an intermediary metabolite of styrene) caused greater increases in GGT and LDH at lower doses (300 mg/kg) and after a shorter time period than did styrene. R-SO was more toxic than S-SO (Gadberry et al., 1996). In addition, 4-vinylphenol (4-VP), a ring-oxidized metabolite of styrene, is about 10-fold more potent than SO at inducing pneumotoxicity in mice as measured by increased lactate dehydrogenase and cells in bronchoalveolar lavage fluid, following ip administration (Carlson, 2002). Further, 4-VP caused extensive cellular damage in the terminal bronchioles as evidenced by exfoliation of epithelial cells into the lumen of the bronchioles leaving a very flattened bronchiolar surface (Carlson, 2002), similar to that reported for naphthalene (Plopper et al., 1992). Inhibiting the metabolism of 4-vinylphenol by inhibitors of either CYP2E1 or CYP2F2 greatly reduced the hepatotoxicity and pneumotoxicity from 150 mg/kg ip (Carlson, 2002). 4-vinylphenol is equally pneumotoxic in

CYP2E1-knockout mice and wild-type (Vogie et al., 2004), providing further evidence of the role of CYP2F2. Using selected CYP inhibitors, Carlson (2004) found that several CYPs contributed to the metabolism of 4-vinylphenol in CYP2E1-knockout mice, but CYP2F2 was most important in the lung. Thus, the toxicity is not caused by 4-vinylphenol, but by a further metabolite. Following two weeks of ip administration of 4-vinylphenol, hyperplasia was seen in the terminal bronchioles of mice from daily doses of 6, 20, or 60 mg/kg/day, but not at 2 mg/kg/day. No lung toxicity was seen in rats at doses up to 60 mg/kg/day. Thus lung toxicity has been demonstrated in mice from styrene, SO and 4-VP, with 4-VP being by far the most potent.

CD-1 mice examined immediately at the end of a six hour inhalation exposure to 40 or 160 ppm styrene had multifocal necrosis and cell loss in bronchiolar epithelium, which was not evident 18 hours later (Green et al., 2001a). Upon repeated exposures, there was decreased cytoplasmic staining of Clara cells and cell crowding in the terminal bronchioles (Cruzan et al., 1997). With longer duration of exposure, hyperplasia of the terminal bronchioles, sometimes extending into the alveolar ducts, occurred. With long-term (lifetime) exposure, lung tumors, primarily benign, were also reported for mice. Lung histopathologic effects have been reported for mice exposed to levels as low as 20 ppm styrene for two years (Cruzan et al., 2001). Effects in alveolar cells were not seen in any of these studies (Cruzan et al., 1997, Cruzan et al., 2001; Green et al., 2001a).

CD-1 and B6C3F1 mice have been exposed to styrene vapor in a series of studies at concentrations from 15 to 500 ppm, 6 hours/day, from 1 to 14 days (Cruzan et al. 1997; Green et al. 2001a). These experiments consistently showed cell crowding, decreased staining and increased cell replication in the Clara cells of the mouse bronchiolar epithelium. Increases in cell replication were seen at dose levels of 40 ppm and above after 3 days (Green et al., 2001a). In a 13 week subchronic study, CD-1 mice exposed to 0, 50, 100, 150 and 200 ppm styrene had changes of the bronchiolar epithelium characterized by decreased eosinophilia and focal crowding of nonciliated cells in bronchioles of mice exposed to 100 ppm and above. Increased labeling (by BrdU) of Clara, but not type II, cells was present after 2 weeks, to a limited extent after 5 weeks, but not at the end of 13 weeks (Cruzan et al., 1997). Although cell proliferation was not measured, similar histopathological findings were reported by Roycroft et al (1992) in B6C3F1 mice exposed to up to 500 ppm styrene for 13 weeks.

In a two-year inhalation chronic toxicity/oncogenicity study, groups of 70 CD-1 mice/sex were exposed to 0, 20, 40, 80 or 160 ppm styrene, 6 hours/day, 5 days/week for up to 2 years (Cruzan et al., 2001). Based on observations from interim necropsies at 52 and 78 weeks and the terminal necropsy at 104 weeks, the lung effects progressed from decreased eosinophilia of the epithelium of the terminal bronchioles to hyperplasia of the terminal bronchiolar epithelium, and finally, to hyperplasia extending into alveolar ducts. With increasing duration, the exposure concentration at which effects were seen also decreased, such that at 104 weeks, mice in all dose levels were affected.

The cells comprising the areas of hyperplasia in the terminal bronchioles stained immunohistochemically with high intensity for CC10, a protein found in normal Clara cells. Immunostaining for surfactant A, a protein more characteristic of normal alveolar Type II cells, was only rarely and faintly present (Cruzan et al., 2001). Electron microscopy of hyperplastic epithelium of the terminal bronchioles identified the predominant cell type as the Clara cell

(Mullins, 1998). Decreased numbers of intracellular organelles, likely the secretory granules, were apparent in some of the Clara cells and may correlate with the decreased eosinophilia noted in the terminal bronchiolar cells.

Adverse effects in the lung were not reported in any of four gavage oncogenicity studies in various strains of mice (NCI, 1979a,b; Ponomarkov and Tomatis, 1978), although two of the four studies reported equivocal increases in lung tumors. However, it is not clear how thoroughly the lungs were examined since inhalation was not the route of exposure. In a more recent study of lung effects, an increase in mouse Clara cell proliferation was seen when styrene was administered orally by gavage for 5 days at doses of 100 or 200 mg/kg/day, but not at 10 mg/kg/day (Green, 2001a). However, no morphological changes were seen in this study at any of the dose levels.

No morphologic or cell proliferation effects were seen in the alveolar region in any of the mouse studies. Toxic effects in Clara cells have been reported following oral and inhalation exposure to styrene. Overall, these findings indicate that the Clara cell is the target cell for the toxic action of styrene in mouse lungs.

In contrast to the effects observed in mice, there were no styrene-related effects in the lungs of Sprague-Dawley rats (Cruzan et al., 1997) or F344 rats (Roycroft et al., 1992) at concentrations up to 1,500 ppm for 3 months. Three long term chronic toxicity/oncogenicity inhalation studies of styrene have been conducted in Sprague-Dawley rats without identifying the lung as a target organ. These include 0, 600, or 1,000 ppm 6 hours/day, 5 days/week for 18.3 (males) or 20.7 (females) months (Jersey et al., 1978); 0, 25, 50, 100, 200 or 300 ppm, 4 hours/day, 5 days/week for one year (Conti et al., 1988); and 0, 50, 200, 500 or 1,000 ppm 6 hrs/day 5 days/week for 24 months (Cruzan et al., 1998). In the most recent study, pale foci were seen in the lungs of a few female rats exposed to 1,000 ppm styrene for 2 years; histopathologic evaluation identified these as foamy alveolar macrophages and/or cholesterol cleft granulomas. These were judged not to be treatment-related lesions of lung tissue (Cruzan et al., 1998) and are not similar to lesions seen in the terminal bronchioles of mice (Cruzan et al., 2001).

Although rats have been given styrene orally in many studies, lung lesions have not been reported. Gavage studies were conducted at dose levels as high as 2,000 mg styrene/kg (NCI, 1979a); 700 (males) or 350 (females) mg/kg styrene (NCI, 1979b); 5 days/week, for 78 or 79 weeks followed by 27 - 29 weeks without dosing prior to termination; and up to 250 mg/kg, 4-5 days/week, for 52 weeks followed by 52 weeks of observation (Conti et al., 1988). No lung effects were associated with two years of ingestion of styrene in the drinking water at 250 ppm (14 mg/kg/day for males and 21 mg/kg/day for females, dose limited by styrene solubility in water) in Sprague-Dawley rats (Beliles et al., 1985).

Cell proliferation studies of the cells of the terminal bronchioles and type II alveolar cells from Sprague-Dawley rats exposed to levels up to 1,500 ppm styrene for 2, 5 or 13 weeks (Cruzan et al., 1997) or 500 ppm for 1, 5, 6 or 10 exposures (Green, 2001a) have also been reported. There were no effects present on labeling indices for either bronchiolar cells or alveolar type II cells of rats at any exposure level or time point.

Although multiple studies discussed above found no styrene-related effects in the lungs of rats, Coccini et al. (1997) reported thickened alveolar septae in rats exposed to 300 ppm styrene, 6 hours/day, 5 days/week, for 2 weeks. Electron microscopic evaluation detected a few alveolar type II cells or bronchiolar cells with dilated endoplasmic reticulum. Increased thickness of the alveolar septae was reported to be due to the presence of collagen fibrils. The ultrastructural effects were reported to be almost completely reversed three weeks after cessation of exposure with only mild alterations in the cytoplasm of some bronchiolar or type II cells still present. Similar effects were reported from intraperitoneal injection of 40 or 400 mg styrene/kg for three days, but not at 4 mg/kg/day (Coccini et al., 1997). The reason for these findings by Coccini and coworkers is unknown, but the numerous studies with much greater exposure concentrations and longer durations suggest that these effects, if present, did not progress, but regressed.

Clara cells differ significantly between laboratory rodents and humans, and even among rodent species, in both number and structure. In mice they are numerous and are spread throughout the airways, whereas in rats they are significantly fewer in number, particularly in the terminal bronchiolar region. In human lung, Clara cells are rare, being found in small numbers in the distal bronchioles. They also differ morphologically, mouse lung Clara cells contain abundant smooth endoplasmic reticulum, while the human Clara cell is apparently largely devoid of these membranes (Smith, 1979, Plopper et al. 1980a,b; Pinkerton et al. 1997). This difference in morphology is consistent with the observed differences in cytochrome P-450 activity, the smooth endoplasmic reticulum being the membranes where the cytochromes P450 enzymes are heavily localized.

Thus, in contrast to mice, lung toxicity in rats from styrene exposure is either nonexistent, or very subtle compared to the overt toxicity in mice. In addition, in rats styrene does not induce progressive lesions based on histopathologic evaluation of eight long-term studies. Furthermore, lung toxicity has not been reported in humans.

2.1.2. Nasal Olfactory Epithelium

A single exposure of CD-1 mice to 80 ppm, but not 40 ppm, styrene resulted in early atrophy/degeneration of olfactory epithelial cells with dilatation of Bowman's glands. Continued exposure resulted in replacement of olfactory cells by ciliated columnar cells (respiratory-like), Bowman's gland hyperplasia and debris (Cruzan et al., 2001). After 13 weeks of exposure at 100 - 200 ppm, nearly all CD-1 mice had atrophy of olfactory epithelium and dilatation of Bowman's glands (Cruzan et al., 1997). At 50 ppm, approximately half of the mice were similarly affected. Fewer mice at each dose level also had atrophy of olfactory nerve fibers and replacement of olfactory cells with respiratory cells. After 2 years of exposure at 20, 40, 80 or 160 ppm, treatment-related changes were also present in the nasal passages in male and female mice (Cruzan et al., 2001). The major findings were respiratory metaplasia of the olfactory epithelium and changes of the underlying Bowman's glands, including dilatation, respiratory metaplasia, epithelial hyperplasia, eosinophilic material/debris, cholesterol clefts, atrophy of nerve fibers and turbinate bone changes. The lesions showed progression with time.

Styrene-related histopathological changes were seen in the nasal olfactory epithelium of male and female CD (Sprague-Dawley) rats exposed to 1,000 or 1,500 ppm for 13 weeks (Cruzan et

al., 1997). At 500 ppm, two of 20 rats were affected; no differences from controls were seen at 200 ppm. After two years of exposure, olfactory lesions (atrophy/degeneration in epithelium, prominent Bowman's glands, atrophy/dilatation/hypertrophy/hyperplasia of Bowman's glands) were present at exposure concentrations of 50 to 1,000 ppm in CD rats (Cruzan et al., 1998).

Based on these findings, the nasal olfactory effects in mice occurred at lower concentration levels and were more severe than in rats. In humans, a limited study found no increase in nasal symptoms or histology scores in 11 reinforced plastics workers exposed to 47-59 ppm styrene for 1 to 16 years (Odkvist et al., 1985). In addition, olfactory function was not decreased in a group of 52 reinforced plastics workers exposed to 30-60 ppm styrene for at least 4 years (Dalton et al., 2003).

2.2. Metabolism and Biokinetics: Differences among mouse, rat and human

2.2.1. Urinary Metabolites

The major metabolic pathways of styrene in mouse, rat and human are shown in Figure 1. In mice, there are four metabolic pathways for styrene:

1. Oxidation of the side chain of styrene by P450s to styrene oxide (SO), which is further converted by an epoxide hydrolase (EH) to the styrene glycol, with subsequent conversion to acidic metabolites, such as mandelic, phenylglyoxylic, and hippuric acids;

2. Oxidation of the side chain of styrene by P450s to styrene oxide (SO) which is further converted by glutathione transferase (GST) to GSH adducts which are eventually excreted as mercapturic acids in the urine;

3. Formation of phenylacetaldehyde either by rearrangement of SO or P450 conversion of styrene to phenylethanol, which is followed by conversion to phenylacetic and phenylaceturic acids;

4. Oxidation of the benzene ring of styrene (possibly leading to the formation of 4-vinylphenol and possibly additional oxidation of the side chain).; these intermediates may result in ring opening which can lead to further oxidation to acids or conjugation with GSH.

Differences in styrene metabolism among humans, rats and mice have been studied by examining differences in patterns of urinary metabolites. These differences are summarized below and reflect data from rats and mice exposed to 125, 250 or 500 ppm styrene for 6 hours (Sumner et al., 1995) and humans exposed to 50 ppm styrene for 2 hours (Johanson et al., 2000).

Species Differences in Styrene Metabolism as Indicated by Urinary Metabolites

Species	Metabolic Pathway			
	SO-EH	SO-GSH	PAA	Ring
B6C3F1 Mouse	49-52%	33-35%	12-17%	4-8%
CD-1 Mouse	51-59%	20-27%	21-22%	4-8%
F344 Rat	68-72%	23-26%	3-5%	<1%
Human (2 - 4 hours)	95%	ND*	5%	ND
Human (4 - 9 hours)	100%	ND*	ND	ND

SO-EH = Styrene oxide and epoxide hydrolase to mandelic acid, etc.;
SO-GSH = glutathione conjugates;
PAA = products derived from phenylacetaldehyde, such as phenylacetic and phenylaceturic acids.;
Ring = ring-opened compounds with or without GSH conjugation.
ND = Not Detected.
*Other authors have reported trace amount of GSH conjugates in human urine, which were below the limits of detection in this study; see Manini et al, 2000; Maestri et al., 1997; Ghittori et al., 1997.

Figure 1. Styrene Metabolic Pathways
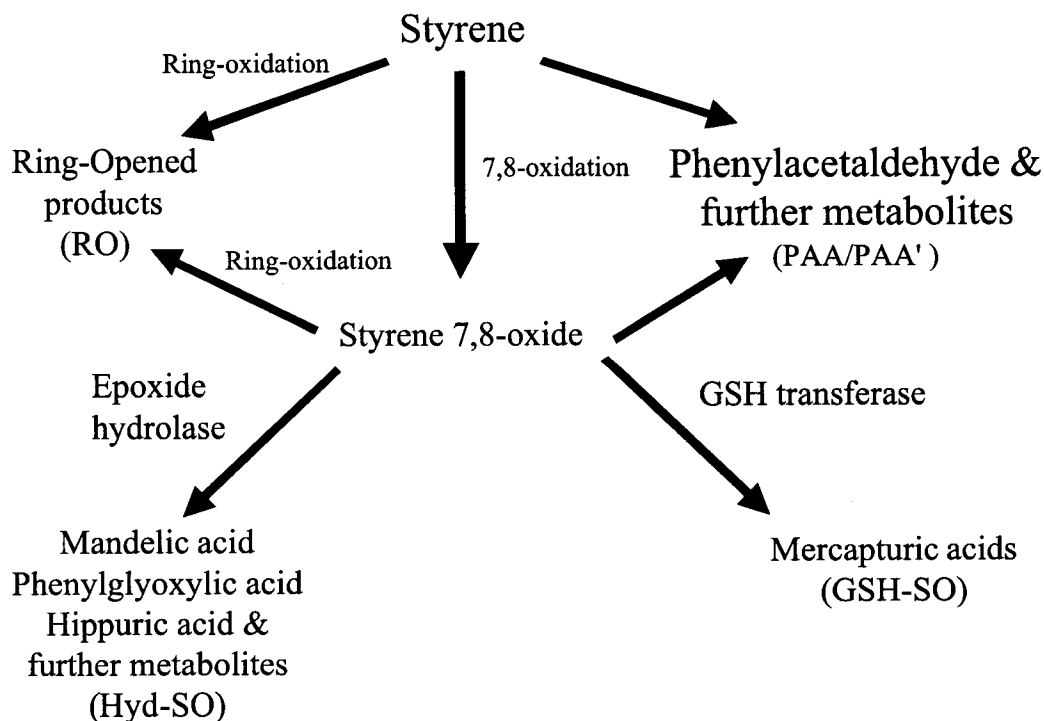
The urinary metabolite data indicate important qualitative differences in metabolism among the species. The epoxide hydrolase pathway accounts for the greatest percentage removal of SO in rats and mice, but the use of GSH conjugation is also an important metabolic pathway for removal of styrene oxide for them. The major differences between rats and mice are in the proportion of styrene metabolism through the PAA (~4% vs. ~20%) and ring-opened (<1% vs. ~6%) pathways. Based on urinary metabolites, humans metabolize styrene almost exclusively via the epoxide hydrolase pathway, with less metabolized via the PAA pathway than in either rats or mice. No more than trace amounts of GSH conjugates or ring-opened metabolites occur in humans exposed to styrene. Thus, the ring oxidation and PAA pathways are used much more in the metabolism of styrene in mice than in rats; these pathways play very small roles in styrene metabolism in humans. These data demonstrate that there are major species differences, both qualitative and quantitative, in the overall metabolism of styrene, which leads us to ask if there are target organ differences in styrene metabolism that might explain differences in toxic response.

2.2.2. Hepatic metabolism of styrene

Based on physiologically-based pharmacokinetic (PBPK) models (Csanady et al., 1994; Sarangapani et al., 2002), metabolism of styrene by liver is probably the most important for removing styrene from an exposed animal and is largely responsible for the body burden/blood level of SO. However, blood levels of SO were much higher in rats at non-tumorigenic concentrations (1,000 ppm) than in mice at levels (40-160 ppm) that caused increased lung tumors (Cruzan et al., 2001). Since blood levels of SO do not correlate with increased incidence of lung tumors, it would appear that toxic effects of styrene in mouse lung tissue are likely the result of localized tissue metabolism of styrene and/or that SO is not the proximate toxicant.

2.2.3. Pulmonary metabolism of styrene

Various studies have shown that microsomal preparations from mouse lung can metabolize styrene to styrene oxide and ring-oxidized metabolites (Carlson 1997a,b, 2000, 2002; Green et al. 2001a). When isolated rat and mouse Clara cell enriched fractions were compared for styrene metabolizing activity, the total amount of SO (pmol / 10^6 cells / min) formed was about five-fold higher in Clara cells from mice than from rats (Hynes et al, 1999). Furthermore a comparison of the ratio of the formation of R-SO to S-SO shows that the mouse Clara cells produces about three times more of the R-enantiomer than the S-enantiomer, while rat produces more of the S-enantiomer (Hynes et al., 1999). This species difference in production of the styrene oxide enantiomers means that mouse Clara cells produce about 15-times more R-SO enantiomer than rat Clara cells. This is important because Gadberry et al. (1996) demonstrated that the R-enantiomer is a more potent pneumotoxicant and hepatotoxicant than the S-enantiomer.

Of the various cytochrome P450 isozymes present in mouse lung tissue, CYP2F2 and CYP2E1 have been identified as being the most important in the pulmonary metabolism of styrene to SO (Carlson 1997b; Green et al. 2001a). Other cytochromes, notably CYP1A and CYP2B appear to play little or no role in the biotransformation of styrene in mouse lung (Carlson et al., 1998; Hynes et al., 1999).

In more recent research, incubation of hepatic microsomes from CD-1 mice with styrene resulted in the formation of SO, but the formation of 4-VP was not detected (Carlson et al., 2001). The authors concluded that this could be because it was not formed, or because it was rapidly metabolized to further metabolic products. They reported that 4-VP readily disappeared when incubated with microsomes from rat and mouse liver and lung in the presence of NADPH, indicating oxidative metabolism. Mouse liver was about three times as active as rat liver and mouse lung about eight times as active as rat lung. Further studies indicated that CYP2E1 and CYP2F2 were important in the metabolism of 4-VP in mouse liver, and that CYP2F2 was more important in the metabolism of 4-VP in mouse lung than was CYP2E1, similar to the metabolism of styrene to SO. They were not able to identify the metabolite(s) formed, but lack of UV absorbance suggests the metabolites are not aromatic; i.e., they may be ring-opened.

The relationship between P450-mediated styrene metabolism and pulmonary toxicity in the mouse has been demonstrated by Green et al. (2001a). Using bronchiolar cell replication as a marker of toxicity, mice treated with 5-phenyl-1-pentyne, an inhibitor primarily of CYP2F2 and to a lesser extent of CYP2E1, showed no evidence of lung cytotoxicity or increased cell replication when exposed to styrene at 160 ppm 6 hours/day for 4 days. In comparison, mice not treated with 5-phenyl-1-pentyne showed evidence of cell necrosis and loss of cells, believed to be Clara cells, with subsequent increases in cell replication. The results demonstrate that Clara cell toxicity and cell replication seen in mice following treatment with styrene is associated with the metabolism of styrene by CYP2F2 and/or CYP2E1. It has been postulated (Green et al., 2001b) that styrene is oxidized primarily by CYP2F2 in tissues that produce high R:S-SO ratios because: 1. inhibition by 5-phenyl-1-pentyne (more potent inhibitor of 2F2 than 2E1) prevents a toxic response, and 2. purified human CYP2E1 (expressed by E.coli) generated an R:S-SO ratio of 0.48. The most recent data also indicate that styrene toxicity could be mediated through 4-VP (or a further metabolite), which is also metabolized primarily by CYP2F2 in mouse lung.

Although there are only limited published data, human pulmonary tissue does not appear to be particularly effective in metabolizing styrene to SO. Nakajima et al. (1994a,b), estimated styrene metabolism in human lung microsomes of 0.0065 nmol/min/mg protein, which is about 100-fold lower than the activity of rat microsomes. Carlson et al. (2000) using microsomes from eight human samples, found only one sample expressing any styrene metabolizing activity (0.088 nmol/min/mg protein) while the other seven samples were devoid of styrene metabolizing activity. All eight human lung samples did however possess P450 metabolizing activity as indicated by their ability to metabolize benzene, which is metabolized in mouse lung by CYP2E1 and CYP2F2. In a similar investigation, Filser et al. (2002) failed to find any styrene metabolism in human lung homogenates even though P450 activity was detected using ethoxycoumarin as a substrate. Using human lymphoblastoid cell cultures that express CYP 2F1, no conversion of styrene to SO could be measured, although these cells do metabolize naphthalene (Yost and Carlson, personal communication).

We note that the CYP2F family contains similar enzymes from different species, but the studies outlined above suggest they may have somewhat different abilities to metabolize styrene. CYP2F1 is found in human tissues, but it does not appear to metabolize styrene. CYP2F2 is found in mice; it readily metabolizes styrene. CYP2F4 is found in rats (particularly nasal tissue); it readily metabolizes styrene.

To determine which pulmonary cells are responsible for the metabolism of styrene, Hynes et al (1999) and Carlson (2000) used enriched fractions of Clara cells and type II pneumocytes. Data from these studies showed that essentially all of the metabolic activity was associated with Clara cells with little or none being found in the type II cells in either rats or mice (Hynes et al., 1999). The data showing that styrene was metabolized by Clara cells but not type II pneumocytes is consistent with the cytochrome P450 isozyme pattern in the cells, i.e. Clara cells contain CYP2E1 and CYP2F2 while type II cells do not (Forkert, 1995; Buckpitt et al, 1995). A comparison of the isozyme pattern in mouse and rat lung indicates the levels of both isozymes CYP2E1 and CYP2F2 are higher in the mouse Clara cell as compared to the rat. These metabolic differences are consistent with toxicity differences in that styrene exposure results in toxicity to mouse Clara cells but not type II cells, or rat lung cells.

Using PBPK modeling, Sarangapani et al (2002) concluded that the target tissue concentration of SO is primarily due to local metabolism of styrene. Based on metabolic data from isolated Clara cells (Hynes et al., 1999) the PBPK model predicts that the concentration of R-SO predominates in the terminal bronchioles of mice, while S-SO predominates in rats. The model predicts a maximum level of 2 μM R-SO in the terminal bronchioles of rats due to saturation of styrene metabolism at 500-600 ppm. This is only one-half the level of R-SO achieved in the terminal bronchioles of mice exposed to 20 ppm styrene. The fact that 20-40 ppm styrene caused an increase in lung tumors in mice, while 500 and 1000 ppm did not cause increased lung tumors in rats may be explained by the lack of sufficient R-SO in rat terminal bronchioles. The Sarangapani et al model predicts that at a given airborne concentration, the level of total SO in the terminal bronchioles of mice is approximately 10-fold higher than in the terminal bronchioles of rats and 100-fold higher than in humans. In humans, the maximum concentration of SO in the terminal bronchioles (~0.09μM) is reached at an airborne concentration of 200 ppm; this is the concentration found in the lungs of mice exposed to 0.1 ppm.

The model predictions demonstrate that SO concentrations in terminal bronchioles of rats and mice are largely determined by local tissue metabolism of styrene. They predict that R-SO levels in the terminal bronchioles of rats do not exceed the minimally tumorigenic concentration in mice. Their model also predicts that humans cannot be exposed to a level of styrene that produces this level of SO in the terminal bronchioles. The PBPK modeling was done using R-SO as the surrogate for toxicity because research at that time pointed to CYP2F2 being important and R-SO being produced largely by CYP2F2. More recently it has been demonstrated that CYP2F2 is also responsible for ring oxidation of styrene and that ring-oxidized metabolites are much more toxic than styrene or SO. Current research has not yet identified the ultimate toxic ring-oxidized metabolite; therefore, the PBPK model has not been updated. Based on the available data, it still represents that styrene metabolism toxic metabolites occur to a much greater extent in mouse lung than rat lung and is practically undetectable in human lung.

The pulmonary metabolism studies and PBPK model of styrene lead to the following conclusions:

1. Styrene metabolism is highest in mouse lung tissue, at a lower level in rat lung tissue, and barely detectable in human lung tissue.

2. R-Styrene oxide, the more toxic enantiomer, is preferentially formed in mouse lung, while S-SO is preferentially formed in rat lung. Further, ring-oxidized metabolites are formed to a much greater extent in mouse lung than rat lung.

3. Metabolism of styrene to SO and ring-oxidized metabolites is carried out primarily, if not exclusively, by Clara cells; no styrene metabolism has been detected in type II pneumocytes.

4. Pulmonary toxicity seen in the mouse is caused by metabolite(s) of styrene formed primarily by CYP2F2.

5. PBPK modeling indicates that the maximal concentration of SO in human terminal bronchioles at any exposure level is less than that present in the terminal bronchioles of mice exposed to 0.1 ppm styrene.

2.2.4. Nasal metabolism of styrene

In rats and mice, the uptake of styrene in the upper respiratory tract is partly dependent on metabolism of styrene (Morris, 2000). When present in the air at 5 ppm, up to 40% of styrene was taken up in the isolated upper respiratory tract of normal rats and mice. The percent absorbed decreased with increasing concentration, demonstrating saturation of metabolism. Saturation of uptake occurred at a lower airborne concentration of styrene in rats than in mice, indicating a greater metabolic capacity in mice than in rats. In rats and mice pretreated with metyrapone (a P450 inhibitor), uptake of styrene was about 10% of the airborne amount, regardless of concentration, between 5 and 200 ppm.

Nasal olfactory epithelium in both rats and mice produce SO from styrene at about the same rate with an R:S ratio of about 3, indicating the primary role of CYP2F2 in mice and 2F4 in rats. Nasal respiratory epithelium from rats and mice produced SO at about half the rate of olfactory epithelium. In contrast, production of SO was not detected in nine human nasal samples (Green et al., 2001b). Olfactory epithelium from rat was more efficient at removing SO via epoxide hydrolase than from mice (Vmax ratio about 10x; Green et al., 2001b). In both rats and mice, the Km for epoxide hydrolase was much lower in respiratory epithelium than in olfactory epithelium, especially for R-SO. Human nasal tissues possessed epoxide hydrolase and GSH transferase activity for both R- and S-SO (Green et al., 2001b). The authors concluded that the differential nasal toxicity between rats and mice could be explained by extensive metabolism of styrene in both species, but a more effective removal of SO in rats than in mice. They further concluded that styrene is unlikely to be toxic to human nasal epithelium.

Prior administration of 5-phenyl-1-pentyne, a cytochrome P450 inhibitor, to mice exposed by inhalation to 40 and 160 ppm styrene, 6 hours/day, for three days protected against cellular damage in the nasal olfactory epithelium, demonstrating that the toxic entity is derived from the cytochrome P-450 metabolism of styrene (Green et al,. 2001b).

2.2.5. Evaluation of Metabolic and Biokinetic Data

The metabolic and biokinetic data lead to the following conclusions:

1. There are significant species differences in the activities of styrene and SO metabolizing enzymes. In those tissues with high levels of CYP2F2 (mouse lung Clara cells, rat and mouse nasal olfactory epithelium), R-SO is the main first metabolite from styrene and tissue damage is found.

2. The toxicity of styrene in lung and nasal tissues is caused by one or more metabolites derived from oxidation of styrene by CYP2F2 which include R-SO and 4-VP or derivatives from 4-VP.

3. The concentration of SO in lung and nasal tissue is mainly due to the local metabolism of styrene. Exposure due to the presence of SO in the blood is of little importance.

4. Differences in nasal toxicity from styrene in rats and mice are consistent with a greater ability of rat nasal tissue to remove SO, especially R-SO, by epoxide hydrolase.

5. Little if any SO is formed in human lung or nasal tissue, but epoxide hydrolase is very active in both human lung and nasal tissue to remove any small amounts formed or that migrates into the cell from the blood. Human lung is incapable of producing sufficient SO to achieve the bronchiolar concentration produced in mice at 0.1 ppm. Thus, no lung or nasal toxicity is expected in humans from styrene exposure.

2.3. DNA Adduct Formation *in Vivo* after Styrene Inhalation Exposure in the Mouse and Rat

Styrene is metabolized to SO, a biologically reactive metabolite. A number of *in vitro* and *in vivo* studies have demonstrated SO adducts to proteins and DNA (IARC, 1994a, b). Therefore, it is important to determine if styrene exposure results in an increased target organ burden of DNA adducts. Low levels of N-7- and O6-guanine DNA adducts were reported in mice exposed to styrene by ip injections of 29 to 450 mg/kg (Pauwels et al., 1996). The authors reported adduct levels corresponding to approximately 15 per 10^8 nucleotides in liver and lung using a ^{32}P post-labeling technique. Otteneder et al. (1999) commented that this method was not as sensitive as claimed by the authors and results less than 30 adducts per 10^8 nucleotides by this method should be viewed with caution. Otteneder et al. (1999) reported that no adducts of O^6-hydroxyethylphenyl guanine were detected (limit of detection 30 per 10^8 nucleotides) using this same ^{32}P-postlabeling assay in mice which had received 1, 5, 6, or 10 six-hour inhalation

exposures to 160 ppm styrene (five days/week). In addition, Otteneder et al. (1999) reported 70 O^6-guanine adducts per 10^8 nucleotides in livers of female Sprague-Dawley rats exposed to 1000 ppm styrene for 2 years.

In the most recent experiment using [ring-U-^{14}C]- styrene, mice were exposed by inhalation to 160 ppm and rats to 500 ppm for six hours (Boogaard et al., 2000). DNA adducts were determined both at the end of the exposure or 42 hours later. A high specific activity styrene (52 mCi/mmol) was used so that chromatographic analysis would reveal the presence of any adducts present at levels greater than 0.1 adducts per 10^8 nucleotides. The N7-guanine adduct was the most prevalent in rat liver; it was present at 3.2 adducts per 10^8 nucleotides at the end of the 6 hour exposure and at 2.1 adducts per 10^8 nucleotides 42 hours later. Other adducts had likewise diminished and some were not detectable at 42 hours. In rat lung, adduct levels were lower than in rat liver; the N7-guanine adduct was present at 1 adduct per 10^8 nucleotides at the end of the 6 hour exposure and at 0.5 adducts per 10^8 nucleotides 42 hours later. In mouse liver, the N7-guanine adduct was present at <1 adduct per 10^8 nucleotides at the end of the 6 hour exposure and at 3 adducts per 10^8 nucleotides 42 hours later. In mouse lung, the N7-guanine adduct was present at 1 adduct per 10^8 nucleotides at the end of the 6 hour exposure and at 0.5 adducts per 10^8 nucleotides 42 hours later, the same as in rats. Three unidentified adducts were present in mouse liver at levels up to 4.7 adducts per 10^8 nucleotides at the end of the exposure; the one present in greatest concentration increased to 12 adducts per 10^8 nucleotides 42 hours later. No increase in adducts was found in mouse lung compared to rat lung. These data indicate that increased lung tumors in mice are not accompanied by an increase in DNA adducts in mouse lung cells. In addition, the authors reported that DNA adducts were not higher in mouse lymphocytes than in lung cells, implying that SO migrating from blood was not the major cause of DNA adducts in lung cells.

The Covalent Binding Index (CBI) as defined by Lutz (1979) was found to be similar for both species and organs: approximately 0.3 in liver and lung of rats and mice; at 42 hours CBI values in liver were 0.14 and 0.44 for rats and mice, respectively. These values confirm the earlier estimates made by Cantoreggi and Lutz (1993) that styrene (and its metabolites) reactions with DNA *in vivo* are very limited.

In summary, all DNA binding studies with styrene in rats and mice have shown no increase in adducts in mice compared to rats or in mouse lung (tumor response) compared to liver (no tumor response). Thus, the DNA adduct data suggest that lung tumors in mice are not due to increased DNA adduct formation in the target cells. Moreover, the very low CBI values calculated in these studies are consistent with the hypothesis that tumor formation in mice exposed to styrene is unlikely to be associated with a primary genotoxic event.

2.4. Genotoxicity of Styrene

2.4.1. Mutagenicity

The mutagenicity of styrene has been tested in multiple assays of various strains *of Salmonella typhimurium* with varying results. In strains TA 98, TA 1537, and TA 1538, which detect frameshifts, styrene has not been mutagenic with or without S9 activation systems in 13 to 15

different tests (Milvy and Garro, 1976; Vainio et al., 1976; Stoltz et al., 1977; deMeester et al., 1977, Watabe et al., 1978; Busk et al., 1979; De Flora et al., 1979; Florin et al., 1980; deMeester et al., 1981; Dunkel et al., 1985; and Brunnemann et al., 1992).

Of the 15 assays of styrene in strain TA 100 and 12 in strain TA 1535 (which detect base substitutions) in the absence of S9 (see above for references), only deMeester et al. (1981) reported increased mutations; however, the level of revertants was only slightly above the criteria of twice background in TA 100. DeMeester et al. (1981) reported there was a dose-related increase in revertants in TA 1535, but this increase is not obvious from the data reported.

In the presence of S9, deMeester et al. (1981), Vainio et al. (1976), and Watabe et al. (1978) reported increased revertants in strain TA 100 and/or 1535. Ten to 12 other studies, using approximately the same styrene concentrations reported no increase in revertants in the presence of S9 in strains TA 100 or TA 1535.

In the presence of S9, deMeester et al. (1981) reported increased revertants in strain TA 100, but the levels were less than in the absence of S9. They also reported increased revertants for strain TA 1535 in the presence of S9 at 8.0 and 10.6, but not at 13.3 ppm in air. The methods used by deMeester et al. (1981) are unclear because they give concentrations in the low ppm range, but say they used an atmosphere of 24% (i.e., 240,000 ppm) styrene. Given the vapor pressure of styrene, levels of 240,000 ppm are not possible. These findings were in apparent contradiction with those reported in 1980, by the same research group (Poncelet et al., 1980). Poncelet and coworkers reported positive results for styrene with S9 in TA 1535 in the plate test, but negative results with other treatment conditions, such as the pre-incubation test, bacterial fluctuation test and incubation of plates in gaseous atmosphere.

Vainio et al. (1976) also reported increased revertants from styrene exposure in the presence of S9 in strains TA 100 and TA 1535. The authors showed that the mutagenicity of styrene oxide (SO), the presumed mutagenic metabolite of styrene, was enhanced in the presence of either 3,3,3,-trichloropropene oxide (TCPO, an inhibitor of epoxide hydrolase) or diethylmaleate (an inhibitor of GSH transferase) both of which limit the detoxification of SO. In contrast, there was no enhancement of mutagenic activity when styrene was incubated with S9 and either of the two inhibitors of SO detoxification.

In contrast to Vainio et al. (1976), Watabe et al. (1978) reported styrene to be negative in strain TA 100 in the presence of S9 and absence of TCPO, but positive when styrene was incubated with TA 100 in the presence of both S9 and TCPO.

Styrene was reported not mutagenic at the HGPRT locus in V79 Chinese hamster cells up to the maximum concentration tested, 51 mM, in the presence of an S9 metabolic activation system (Loprieno et al., 1976). Beije and Jenssen (1981) also studied the mutagenicity of styrene in V79 Chinese hamster cells at the HGPRT locus but used isolated perfused rat livers as the metabolic activation system. Styrene was not mutagenic in V79 cells without activation. The authors reported a very weak effect when rat liver was used as the activating system. They reported a less than doubling of the mutant frequency in two tests, but not in a third identical test.

In a single study with *Saccharomyces cerevisiae*, styrene induced gene conversion and reverse mutation, using logarithmically growing yeast cells, which possess enough monooxygenase to actively metabolize indirect mutagens (Del Carratore et al., 1983). Styrene did not induce forward mutations in *Schizosaccharomyces pombe* in the presence or absence of metabolic activation (Loprieno et al., 1976; Bauer et al., 1980). The SOS Chromotest in *Escherichia coli* was reported positive without dose response in one test (Glosnicka and Dziadziuszko, 1986) and negative in the presence of metabolic activation at a ten-fold higher dose in another test (Brams et al., 1987). In a single study with *Drosophila melanogaster* a statistically significant increase in the frequency of recessive lethal mutation was observed (Donner et al., 1979). Styrene produced no effect on induction of sex-chromosome non-disjunction in Drosophila and was non-active overall in the somatic w/w+ assay of Drosophila.

On 3 of 4 occasions, nine styrene-exposed lamination workers from Bohemia (measured exposures from 5 to 50 ppm) had a slightly higher frequency of *hprt* mutant cells than seven non-exposed workers in the same factory (means = 17.5×10^{-6} vs. 15.7×10^{-6}) studied simultaneously, but the differences were not statistically significant. The level of mutations was significantly greater than in eight persons (presumed to be without styrene exposure) from a research institute in Prague on the one occasion this comparison was made (mean 11.8×10^{-6}). The level of mutations did not correlate with the level of DNA adducts or DNA strand breaks (Vodicka et al., 1995). No measurement of exposure to SO or other chemicals was reported. Comparison of Glycophorin A somatic cell mutation frequencies in erythroid progenitor cells in 47 Finnish lamination workers compared to 47 unexposed matched controls demonstrated no increase in _/N variant cell frequency, indicating no gene activating mutations, including point mutations or deletions (Bigbee et al., 1996). The authors did report an increase in N/N variant cell frequency among workers exposed to greater than 30 ppm styrene; a statistically significant dose response was reported relative to atmospheric styrene concentration, but not based on urinary metabolites of styrene (Bigbee et al., 1996). N/N variants reflect a mechanism of mitotic recombination.

In summary, in lower eukaryotes, plants, V79 cells, and Drosophila, it appears that styrene is either negative or weakly positive in inducing different mutagenic endpoints (reviewed by Preston, 1990). In two human studies, styrene exposure may have been weakly associated with increased mutations. The results of the Ames assays suggest that styrene, if mutagenic, causes deletion type mutations, while the human Glycophorin A assay indicated no deletions or point mutations from styrene exposure. The only mutagenicity tests in lung cells, V79 from hamster, were negative.

2.4.2. Cytogenetics

Chromosome Aberrations

Styrene added to human whole-blood culture was shown to induce chromosome breaks, and aneuploidy in lymphocytes, without exogenous metabolic activation (Linnainmaa et al., 1978a; Pohlova et al., 1985; Jantunen et al., 1986). The authors suggest this is mediated by the presence of oxyhemoglobin. Increased chromosome aberrations (CA) were reported in Chinese hamster lung cells with S9 from methylcholanthrene-induced rats (Matsuoka et al., 1979) and in *Allium cepa* without metabolic activation (Linnainmaa et al., 1978b).

In contrast to the *in vitro* studies, styrene has not generally increased CA in laboratory animals. Increased CA were reported in only one (Meretoja et al., 1978b) of nine studies (Norppa et al., 1980b; Sbrana et al., 1983; Sinha et al., 1983; Sharief et al., 1986; Kligerman et al., 1992, 1993; Preston & Abernathy, 1993) where styrene was administered at dose levels similar to those used by Meretoja et al. (1978b). Of particular interest relative to lung tumors, Kligerman et al. (1992) found no increase in CA in lung tissue of B6C3F1 mice exposed to 125, 250 or 500 ppm styrene for 14 consecutive days.

A number of human cytogenetic monitoring studies have been carried out on workers exposed to styrene in reinforced plastics processes. Some investigators incubated lymphocytes in TC199, a folate deficient medium that is not recommended for human studies or incubated for inappropriate times (Preston, 1990b). Most authors have suggested that any cytogenetic effects seen in reinforced plastics workers arise from the metabolism of styrene to styrene oxide (SO). Rappaport et al. (1996) concluded that most of the SO circulating in the blood of reinforced plastics workers may originate from atmospheric SO, not from the metabolism of styrene. There are insufficient data to ascertain what relationship, if any, there is between atmospheric styrene and atmospheric SO in reinforced plastics operations. Further, reinforced plastics operations may involve exposure to a number of other chemicals that are known or suspected to cause cytogenetic effects; e.g., cyclohexanone, benzoylperoxide, methylethyl ketone peroxide, dimethylaniline and maleic anhydride (Scott, 1993; Scott & Preston, 1994). None of the studies have measured or estimated the exposure level of any of these agents in the reinforced plastics workers whose lymphocytes were examined for cytogenetic damage.

Of the 23 studies of chromosomal aberrations in reinforced plastics workers, nine reported a significant increase in frequency among styrene exposed workers compared to controls. Chromosome gaps were frequently included as CAs. None of these investigators measured exposure to any agent except styrene. The following authors reported increased CA:

Meretoja et al., 1978a	- 15 workers exposed to up to 300 ppm styrene, 6 controls
Fleig & Theiss, 1978	- 14 workers exposed to from 50 to 300 ppm, 20 controls
Hogstedt et al., 1979	- 6 workers exposed to from 14 to 102 ppm, 6 controls
Anderson et al., 1980	- 36 workers exposed to from 0 to 232 ppm, 37 controls
Camurri et al., 1984	- 41 workers exposed to from 7 to 96 ppm, 30 controls
Dolmierski et al., 1984	- 30 workers exposed to less than 23 ppm, 2 controls
Forni et al., 1988	- 32 workers exposed to from 0.4 to 60 ppm, 32 controls
Tomanin et al., 1992	- 11 workers exposed to from 27 to 104 ppm, 7 controls
Artuso et al., 1995	- 23 workers exposed to from 2 to 323 ppm, 51 controls

The following authors reported no increase in CAs:

Fleig & Theiss, 1978	- 12 workers exposed to from 0 to 47 ppm, 20 controls
Theiss et al., 1980	- 24 workers exposed to from 0.7 to 178 ppm, 24 controls
Watanabe et al., 1981	- 16 workers exposed to from 1 to 211 ppm, 13 controls
Watanabe et al., 1983	- 18 workers exposed to from 40 to 50 ppm, 6 controls
Hansteen et al., 1984	- 18 workers exposed to from 2 to 44 ppm, 9 controls

Nordenson & Beckman 1984	- 15 workers exposed to with average exposure of 24 ppm, 13 controls
Pohlova & Sram, 1985	- 58 workers exposed to from 1 to 236 ppm, 41 controls
van Sittert & deJong, 1985	- 105 workers exposed to from <0.1 to 1.4 ppm, 136 controls
Maki-Paakkanen et al., 1987	- 21 workers exposed to from 8 to 63 ppm, 21 controls
Forni et al., 1988	- 8 workers exposed to from 10 to 48 ppm, 8 controls
Jablonicka et al., 1988	- 11 workers exposed to from 28 to 140 ppm, 11 controls
Hagmar et al., 1989	- 11 workers exposed to from 1 to 38 ppm, 9 controls
Sorsa et al., 1991	- 75 workers exposed to from 1 to 182 ppm, 54 controls
Maki-Paakkanen et al., 1991	- 17 workers with average exposure of 70 ppm, 17 controls

Scott and Preston (1994) concluded that the positive or negative outcome of the various studies bore no relationship to the degree of exposure to styrene and there was no convincing evidence of a dose-response relationship for CA. Bonassi et al. (1996) divided the studies into whether they thought the average exposures were likely to be greater or less than 30 ppm; based on this division Bonassi and coworkers concluded there was greater likelihood of increased CAs in groups with average exposure greater than 30 ppm. Vodicka and coworkers (2004) found no relationship between styrene exposure and chromosome aberrations among 86 reinforced plastics workers exposed an average of 4 years to an average styrene concentration of 20+/-14 ppm.

Micronuclei

Styrene added to human whole-blood culture was reported to induce micronuclei (MN) in lymphocytes, without exogenous metabolic activation (Linnainmaa et al., 1978a). In contrast to the *in vitro* studies, styrene has not generally increased MN in laboratory animals. Increased MN were reported in two (Norppa, 1981; Simula & Priestly, 1992) of seven studies (Kligerman et al., 1992, 1993; Simula & Priestly, 1992; Pentilla et al., 1980) where styrene was administered at similar dose levels. Increased micronunclei were reported after NMRI mice were exposed to 1500 mg/m^3 (~350 ppm) styrene for 7 days, but not after 1, 3 or 21 days of exposure (Vodicka et al., 2001a). These data were not repeated during a re-test (Engelhardt et al., 2003); in the second article the authors indicated the first results must have been spurious. The genotoxicity and cytotoxicity of styrene and 1,3-butadiene were compared in B6C3F1 mice. Mice exposed to 62.5 or 625 ppm butadiene for 8 hours had increased micronuclei, but no cytotoxicity. In contrast, mice similarly exposed to 50 ppm styrene did not have increased micronuclei, but did have cytotoxicity (Leavens et al., 1997). The study authors also reported that a combination of butadiene and styrene did not enhance the frequency of micronuclei.

The frequency of MN was examined in ten human studies, none of which measured exposure to any agent except styrene. Four studies (Meretoja et al., 1977; Hogstedt et al., 1983; Nordenson & Beckman, 1984; Brenner et al., 1991) reported increased MN compared to controls, while six studies reported no increase in MN (Maki-Paakkanen et al., 1987, 1991; Hagmar et al., 1989; Sorsa et al., 1991; Tomanin et al., 1992; Yager et al., 1993). Scott & Preston (1994) and Bonassi et al. (1996) using different evaluation criteria (See description of criteria in CA section above) concluded there was no dose-related increase in MN in the human studies.

2.4.3. DNA Damage and Repair

DNA Repair

Incubation of styrene with rat primary hepatocytes in the DNA repair assay resulted in toxicity at 7.5 or 10 mmol. At lower concentrations, no toxicity or increased DNA repair was seen (Brunnemann et al., 1992). Vodicka and coworkers (2004) demonstrated that styrene exposure increases DNA repair mechanisms in reinforced plastics workers.

DNA Strand Breaks

Belvedere et al. (1984) showed that isolated rat hepatocytes acted more efficiently than S9 and pure microsomes in converting styrene to styrene oxide, suggesting that hepatocytes could be one of the most suitable systems for the study of indirect mutagens *in vitro*. Increased single-strand DNA breaks were reported when freshly isolated rat hepatocytes were exposed to styrene. In addition, the level of alkali-labile single strand breaks was increased in DNA from liver, lung, kidney, testis and brain in mice exposed to a single high intraperitoneal dose of styrene. There were no significant increases in alkali-labile DNA strand breaks in peripheral lymphocytes of rats exposed to styrene. Similarly Kligerman et al. (1993) found no increase in DNA strand breaks in lymphocytes of rats exposed to 500 ppm styrene by inhalation for two weeks. A strong sublinear dose-response increase in DNA strand breaks, using the comet assay, was reported for both styrene and SO administered ip to C57Bl/6 mice. Lymphocyte and liver DNA were most affected, with lesser responses in kidney and bone marrow (no determinations were made on lung DNA; Vaghef & Hellman, 1998). Increased single-strand breakage in workers employed in the RFP industry in Scandinavia has also been reported (Maki-Paakkanen et al., 1991; Walles et al., 1993). N-7-Adducted guanine bases in DNA are markedly susceptible to depurination and strand breaks under alkaline conditions (Damjanov et al., 1973; Lawley , 1963, 1968); thus these assays are not able to distinguish between DNA with N-7 adducts and DNA with strand breaks. Furthermore, single-stranded DNA breaks do not indicate that a mutagenic or clastogenic event has occurred. Vodicka and coworkers (2004) reported a negative correlation between styrene exposure and DNA strand breaks in reinforced plastic workers.

Sister Chromatid Exchanges

Styrene incubated with isolated human lymphocytes showed induction of sister chromatid exchanges (SCEs) (Norppa et al., 1980a, 1983; Norppa &Vainio, 1983; Chakrabarti et al., 1993), in the absence of metabolic activation. Exposure to styrene resulted in increased SCE in eight of nine studies with laboratory animals (Conner et al., 1979, 1980; Sharief et al., 1986; Kligerman et al., 1992, 1993; Simula & Priestly, 1992); only Preston & Abernathy (1993) reported no increase in SCE.

Rappaport et al. (1996) reported that increased SCE correlated with atmospheric SO exposure, but not with atmospheric styrene exposure in a group of 20 reinforced plastics workers where exposure to both chemicals were measured over time. Of thirteen other studies, which did not measure atmospheric SO, two (Artuso et al, 1995; Yager et al., 1990, 1993) reported increased SCE and two (Anderson et al., 1980; Camurri et al., 1983) reported weakly positive results in

reinforced plastics workers compared to controls. Negative results were reported by Meretoja et al. (1978a); Watanabe et al. (1981, 1983); Hansteen et al (1984); Maki-Paakkanen et al. (1987, 1991); Kelsy et al. (1990); Brenner et al. (1991) and Sorsa et al. (1991). Hallier et al. (1994) showed a lower incidence of SCE when the occupational exposure to styrene was reduced. Presumably, the personal protective measures that reduced styrene exposure also reduced exposure to other chemicals in the workplace.

In separate reviews, using different evaluation criteria, Scott & Preston (1994) and Bonassi et al. (1996) concluded there was no dose-related increase in SCE in the human studies.

2.4.4. Genetic Polymorphisms and Styrene

A number of studies have investigated the role of glutathione transferase polymorphisms on SCE or comet assay in styrene-exposed workers, or in *in vitro* studies using human lymphocytes (Ollikinen et al., 1998; Vodicka et al., 2001b; Bernardini et al., 2002, Haufroid et al. 2002; Buschini et al., 2003; Laffon et al., 2003; Teixeira et al., 2004). These studies tend to show that persons with GSTM1 pos genotype show significantly more DNA damage than GSTM1 null individuals, implying glutathione conjugation plays a major role in styrene-related genotoxicity. This is hard to explain since no more than 0.1% of styrene is metabolized by glutathione conjugation in humans. In addition, effects on sister chromatid exchange or comet assay are not dramatically increased in rats and mice compared to humans, despite the fact that ~25% of styrene is conjugated with glutathione.

2.4.5. Overall Evaluation of Styrene Genotoxicity

Styrene and its metabolites have a low reactivity with DNA, which results in either negative or weakly positive results in mutagenicity assays in lower eukaryotes, plants, V79 cells, and Drosophila. *In vitro* assays for chromosome effects have been generally positive using human lymphocytes, Chinese hamster lung cells, or *Allium cepa*. *In vivo* experiments in laboratory animals have generally not resulted in increased chromosomal aberrations or micronuclei. A minority of studies of reinforced plastics workers reported increased chromosomal aberrations or micronuclei. No dose response is evident for micronuclei, but two independent analyses (Scott and Preston, 1994; Bonassi et al., 1996) reached opposing conclusions on whether there was a dose-response for chromosomal aberrations in the human studies. Lack of genotoxic response in mouse lung cells was found as follows: no increase in CA in the lungs of mice exposed to 125, 250 or 500 ppm styrene for 14 days (Kligerman et al., 1992) and no increase in lung tumors in a screening assay in A/J mice (Brunnemann et al., 1992).

In contrast to the generally negative clastogenicity data, a consistently small increase in SCE is seen in animal studies of styrene, but both reviewers of the human SCE studies agreed there was no dose-response increase in SCEs in the human studies.

Increased DNA strand breaks have been reported in animals and workers exposed to styrene; these may be caused by the assay method or related to oxidative damage, rather than direct

genotoxic damage. After several articles reporting increases in a number of genetic related endpoints in reinforced plastics workers, Vodicka and coworkers (2004) reported no clear relationship between styrene exposure and chromosomal aberrations, decreased micronulcei and single strand breaks, and increased DNA repair in reinforced plastics workers. Very low levels of DNA adducts, especially in mouse lung, indicate that induction of mouse lung tumors by a genotoxic mode of action is unlikely.

3. Evaluation of Possible Modes of Action Responsible for the Lung Tumor Formation in the Mouse

This section evaluates the available scientific evidence for genotoxic and nongenotoxic modes of action for the development of lung tumors in mice.

3.1. Genotoxic Mode of Action

The genotoxicity data on styrene are equivocal. Most studies of styrene do not report increased mutations. Chromosomal aberrations and micronuclei were not seen in most animal studies, but several studies indicated a weak induction of sister chromatid exchange. The frequency of alkali labile sites/DNA strand breaks is increased in some studies. A minority of reinforced plastics worker studies indicate increased chromosomal aberrations, micronuclei, or sister chromatid exchange (IARC, 1994a).

Styrene is metabolized, at least in part, to SO, which is mutagenic in several *in vitro* systems (IARC, 1994b). Exposure of humans or animals to styrene results in increased levels of hemoglobin and DNA adducts derived from SO. The R-enantiomer of SO has been shown to be slightly more reactive than the S-enantiomer in the Ames assay (Pagano et al., 1982; Seiler, 1990; Sinsheimer et al., 1993), although Watabe et al. (1981) did not find a difference. Chromosome aberrations were induced in mouse bone-marrow cells in one study by the (S)-enantiomer of SO, but not with the (R)-enantiomer (Sinsheimer et al., 1993). These data indicate that styrene is capable of interacting with DNA in animals and humans.

Two studies suggest a lack of styrene-related genotoxic response in mouse lung cells. Inhalation of styrene at 125, 250 or 500 ppm for 14 days by B6C3F1 mice did not result in increased chromosomal aberrations in lung (Kligerman et al., 1992). No increase in lung tumors occurred in a lung tumor initiation assay in A/J mice (Brunnemann et al., 1992).

3.1.1. Styrene Oxide as Potential Tumorigenic Agent Arising from Metabolism of Styrene

As reviewed above, a large portion of styrene is metabolized to styrene oxide (SO) by laboratory animals and by humans. SO can be detected in the blood of both rodents and humans. SO is a carcinogen in laboratory animals (IARC, 1994). The following section examines the carcinogenicity and mode of action data on SO and the relevance of metabolically generated SO to the evaluation of the carcinogenic potential of styrene in humans.

3.1.1.1. Tumor Data

No epidemiology studies of humans exposed to styrene oxide are available. Although reinforced plastics workers are exposed to SO, as well as styrene and other chemicals, very few measurements of SO exposures have been made and no comparisons of causes of death with SO exposures have been made.

Gavage administration of 275 or 550 mg/kg/dose 3 times/week for 104 weeks of SO to F344 rats produced forestomach papillomas and carcinomas in 50/52 and 50/51 males, respectively, and in 46/52 and 50/52 females, respectively (Lijinsky, 1986). Gavage administration of 50 or 250 mg/kg/dose SO 4 to 5 times/week for 52 weeks (observed to death) to Sprague-Dawley rats resulted in forestomach papillomas and carcinomas in 18/40 and 39/40 males, respectively, and in 11/40 and 38/40 females, respectively (Conti, et al., 1988). Gavage administration of 100-150 mg/kg/dose SO once per week for 96 weeks (observed to 120 weeks) to BDIV rats also resulted in forestomach tumors (Ponomarkov et al., 1984). There were no increases in tumors at sites distant to the site of contact in rats in any of the three studies. In B6C3F1 mice (Lijinsky, 1986), increased forestomach papillomas and carcinomas were reported at doses of 375 and 750 mg/kg/dose of SO three times/week for 104 weeks. In males treated at 375, but not 750 mg/kg/day, there was a higher incidence of liver tumors than in the control mice [12/51 (24%), 28/52 (54%), 15/52 (29%) for 0, 375, and 750 mg/kg/dose, respectively]. In females, the incidence of liver tumors was not different from controls. Because the reported increase in liver tumors occurred only in males and only at the low dose, it appears that oral administration of styrene oxide results in tumors only at the site of contact, the forestomach, in rats and mice.

Dermal administration of SO to Swiss-Millerton (Van Duuren et al., 1963) and C3H mice (Weil et al., 1963) caused no increases in skin tumors or in internal tumors. No inhalation studies of SO have been reported.

3.1.1.2. Mode of Action

The purpose of this section is to examine the mode of tumorigenic action of SO in order to assess the relevance of the application of SO data to the assessment of the tumorigenic potential of styrene. SO is reactive in biological systems; its half-life in fresh rodent blood (*in vitro*) was reported to be 26-34 minutes when added at 10 μg/g blood and 41 minutes when added at 100 μg/g (Langvardt and Nolan, 1991); Kessler et al. (1990) reported half lives in blood of 24.5±3.1 min at SO concentration of 1 to 10 μg/ml and 32±3.5 min in the range of 10 to 100 μg/ml. An earlier study (Bidoli et al., 1980) reporting a half-life in mouse blood of 3.4 minutes is probably not correct since two independent studies using more modern techniques arrived at values that agreed with each other and were quite longer than 3.4 minutes. SO has been shown to react with hemoglobin and DNA *in vitro* and *in vivo*.

3.1.1.3. Genotoxicity

SO is mutagenic in bacteria systems without metabolic activation. It induced base-pair substitution mutations in *Salmonella typhimurium* strains TA1535 and TA 100 (Milvy & Garro, 1976; Vainio et al., 1976; deMeester et al., 1977; Sugiura et al., 1978a; Wade et al., 1978;

Watabe et al., 1978; Watabe et al., 1980; Busk, 1979; El-Tantawy & Hammock, 1980; Yoshikawa et al., 1980; DeFlora, 1981; deMeester et al., 1981; Sugiura & Goto, 1981; Turchi et al., 1981; Brams et al., 1987; Glatt et al., 1983; Hughes et al. 1987; Claxton et al., 1991; Einisto et al., 1993; and Sinsheimer et al., 1993), and forward mutations in the SOS *umu* test (Nakamura et al., 1987). SO induced mutations in *E.coli* strains in the liquid micromethod procedure (Glosnicka & Dziaduszko, 1986; Brams et al., 1987; von der Hude et al., 1990). SO induced forward mutations in *Klebsiella pneumoniae* (Voogd et al., 1981) and *Schizosaccharomyces pombe* (Loprieno et al., 1976). SO induced gene conversion in *Saccharomyces cerevisiae* (Loprieno et al., 1976). The R-enantiomer of SO has been shown to be slightly more reactive than the S-enantiomer in the Ames assay (Pagano et al., 1982; Seiler, 1990; Sinsheimer et al., 1993), although Watabe et al. (1981) did not find a difference. Styrene reaction products with glutathione, cysteine, cysteinylglycine and mercapturic acid did not induce mutations in *Salmonella typhimurium* strain TA 100 (Pagano et al., 1982).

In cultured mammalian cells, SO induced gene mutations at the *hprt* locus in V79 Chinese hamster lung cells (Loprieno et al., 1976, 1978; Bonatti et al., 1978; Beije &Jensen, 1982; Nishi et al., 1984) and *tk* locus in L5178Y (mouse lymphoma) cells (Amacher & Turner, 1982). Incubation of SO with T-lymphocytes from two human donors resulted in increased base-substitution mutations. Frameshift mutations and small deletions were less frequent and splicing mutations were more frequent than in control lymphocytes (Bastlova & Podlutsky, 1996). Exposure of cultured renal (LLC-PK1) cells for 7 weeks to 5μM SO (one-tenth the dose that caused LDH leakage and/or inhibited cell proliferation) resulted in altered cell morphology, but did not result in mutations. Parallel exposure to similar doses of S-(1,2-dichlorovinyl)-L-cysteine, potassium bromate, and cis-platinum increased poly (ADP-ribosyl)ation and increased mutations of the p53 gene (Richter & Vamvakas, 1998). The authors concluded that styrene oxide induces forestomach tumors due to cytotoxicity, not genotoxicity.

Sex-linked recessive lethal mutations were reported in *Drosophila melanogaster* (Donner et al., 1979), however, a dominant lethal test in mice was negative (Fabry et al., 1978). SO did not induce transformation of C3H10T1/2 cells (Male et al., 1985).

Several other styrene metabolites including styrene glycol, hippuric acid, mandelic acid and phenyl glyoxylic acid were reported not to induce bacterial mutagenicity (deMeester, et al, 1977; Milvy and Garro, 1976).

3.1.1.4. Cytogenetics

SO has been reported to increase micronuclei and anaphase chromosome bridges (Turchi et al., 1981), and chromosome aberrations (Fabry et al., 1978) in cultured human lymphocytes.

The *in vivo* cytogenetics data for SO are not as clear. Chromosome aberrations were induced in mouse bone-marrow cells in one study by the (S)-enantiomer of SO, but not with the (R)-enantiomer (Sinsheimer et al., 1993). In two other studies with mice, conflicting results were obtained in that a significant increase in bone-marrow cell chromosome aberrations was reported in one study (Loprieno et al., 1978) at a five-fold lower dose level than was used in another study (Fabry et al., 1978) in which no significant response occurred. No chromosome aberrations were

reported in Chinese hamster bone marrow by ip or inhalation (Norppa et al., 1979). Micronuclei were not induced by SO in bone marrow of mice (Fabry et al., 1978) or Chinese hamsters (Pentilla et al., 1980).

3.1.1.5. DNA Damage and Repair

In cultured mammalian cells, SO induced DNA single-strand breaks, but not double-strand breaks or cross-links (Walles & Orsen, 1983). Recent data showed that human microsomal epoxide hydrolase expressed in mammalian cells has a protective effect against SO-induced DNA damage (as measured by single strand DNA breaks) at SO concentrations up to 100 μM, which gradually diminished by increasing SO concentration up to 1mM, i.e., 1000μM (Herrero et al., 1997). This was in contrast to ethylene oxide (EO); epoxide hydrolase did not have a protective effect against EO-induced DNA damage (Herrero et al., 1997).

Exposure of human white blood cells (WBC) to SO *in vitro* resulted in a decrease in high molecular weight DNA fragments and an increase in low molecular weight fragments (Marczynski et al., 1997b). The response was identical to that produced by hydrogen peroxide, a typical oxidant linked to oxidative stress. The authors concluded that SO causes DNA strand breaks as a result of oxidative stress. The authors suggest that increased single strand breaks seen after styrene exposure could be due to oxidative stress.

Intraperitoneal (IP) administration of SO to C57Bl/6 mice resulted in increased DNA strand breaks in lymphocytes, liver, kidney and bone marrow cells (i.e., all tissues examined); no measure of strand breaks in lung tissue was made (Vaghef and Hellman, 1998). The least effect was seen in bone marrow (4 fold) and the greatest in lymphocytes (16 fold). In all tissues, the effect was greater 4 hours after administration than after 16 hours, suggesting rapid repair of DNA damage. In addition, the authors reported a strong sub-linear dose response with small, if any, increases in DNA strand breaks at doses of 50, 100, or 150 mg/kg SO, but large increases in strand breaks at 200 mg/kg (Vaghef and Hellman, 1998).

SO induces SCEs in cultured human lymphocytes (Norppa et al., 1981, 1983; Lee and Norppa, 1995). Chakrabarti and coworkers (1997) demonstrated a significant inverse relationship between the duration of exposure of cultured human lymphocytes with 100 μM SO and the formation of SCEs; the authors suggest this is due to elevations in DNA repair and in detoxification of SO. Incubation of SO with human lymphocytes from donors who had the glutathione transferase T1 (*GSTT1*) gene resulted in a lower level of SCE than in lymphocytes from donors who lacked the *GSTT1* gene (Ollikainen et al., 1998). The authors suggest these results indicate that reaction with GSH may be important in the detoxification of SO; however, as reviewed in the metabolism section of this document, reaction with GSH plays a very minor role in the detoxification of SO in humans. In contrast to the *GSTT1* gene, the presence or absence of the glutathione transferase M1 (*GSTM1*) gene had no effect on the formation of SCE in cultured human lymphocytes (Uuskula et al., 1995). The presence of absence of *GSTM1* has been reported to be important in the formation of SCE in butadiene exposure. Nishi et al (1984), found SO was much more effective in inducing SCE than point mutations in Chinese hamster V79 cells *in vitro*.

There was no increase in SCE in hamster bone marrow after inhalation or ip administration of SO (Norppa et al., 1979) nor in mouse bone marrow after inhalation (Conner et al., 1982); however, mouse liver cells and alveolar macrophages had a borderline increase in SCE frequencies (Conner et al., 1982).

In vitro reactions of SO with nucleosides and DNA have been reported by several authors. When SO was reacted *in vitro* with salmon testis DNA, (Savela et al., 1986) 82% of the guanine adducts were at the N-7 position. Reaction of a ten-fold excess of SO with DNA resulted in the formation of several adducts, which included N-7 guanine, O6-guanine, and N-2-guanine adducts, as well as 3 adducts that were described as being nucleotides modified by two molecules of SO (Pongracz et al., 1989; 1992; Kaur et al., 1993). These bis-adducts are not likely to be found under typical conditions of exposure of humans or animals to SO or styrene since they were formed only with a 10 fold excess of SO.

Incubation of human embryonal lung (HEL) cells with SO for 3 hours resulted in 3-fold higher levels of N-7-alkylguanine adducts and single strand breaks than incubation for 18 hours (Vodicka et al., 1996), suggesting that DNA adducts and single strand breaks from SO are rapidly repaired. At 3 hours, but not 18 hours, there was a significant correlation between DNA adducts and strand breaks. Based on ^{32}P-postlabeling, the same laboratory has shown that the 8-carbon (terminal) of SO binds preferentially to the N-7 of guanine in Salmon testis DNA to form the β-isomer, compared to the 7-carbon, which forms the α-isomer; the ratio was 63:37 (Kumar et al., 1997). Two diastereomers of the β-isomer of hydroxyethylphenyl-N-7-guanine were separated; one was present in 3-fold greater yield (Kumar et al., 1997). It is not known if the method labels the isomers in different quantities or if they are present in different quantities in the DNA.

Low levels of adducts have been reported in laboratory animals exposed to SO. No adducts were detected in liver of rats following oral administration of radiolabeled SO (240 mg/kg) or in mice (165 mg/kg) after ip administration (Cantoreggi and Lutz, 1992). By pooling DNA from six rats, which improved the limit of detection, Lutz et al. (1993) detected total DNA adducts at a level of 0.4 in 10^7 nucleotides in forestomach DNA (0.04 μmol/mol nucleotide/mmol/kg) from rats administered 250 mg/kg [7-^{3}H]SO by gavage; identification of adducts was not determined. DNA adduct levels were determined by HPLC of the hydrolyzed nucleotides following repurification of the DNA to constant specific radioactivity. Approximately 60% of the radioactivity eluted with normal nucleotides, indicating metabolic incorporation of tritium from SO into normal nucleotide constituents, not adducts of SO to DNA (Lutz, 1995). Byfalt-Nordqvist et al. (1985) reported approximately 40-fold higher DNA adduct levels in mice. Since the DNA was not repurified to constant specific activity, it is not clear if all the SO-radioactivity was bound to DNA. Phillips and Farmer (1994) recommend accepting the levels (0.4 in 10^7 nucleotides) derived by Lutz et al. (1993) as the correct ones.

Incubation of fresh human blood with EO, propylene oxide (PO), or SO demonstrated that SO had the lowest elimination rates; the second-order rate constant for reaction with hemoglobin was 3-fold lower for SO than for EO, and the second order rate constant for reaction with WBC DNA was 20-fold lower than EO (Pauwels and Veulemans, 1998). PO had rate constants for

both hemoglobin and DNA adducts that were about 3-fold lower than for EO. As a result the ratio of reaction to hemoglobin to DNA was 169, 196 and 2113 for EO, PO, and SO, respectively. The authors point out that when SO, but not EO or PO, is given *in vivo*, a lower level of hemoglobin formation (μM adduct/μM epoxide) is seen in rats and mice than is predicted from the *in vitro* reaction; this they attribute to SO being a better substrate for epoxide hydrolase than EO or PO.

3.1.1.6. Cell Proliferation

Lutz's group also reported increased cell proliferation in rat forestomach following oral administration of SO (Lutz et al., 1993; Cantoreggi et al., 1993b) for four weeks at 137, 275 and 550 mg/kg/day. Butylhydroxyanisole (BHA) given at 1 or 2% in the diet caused a similar percentage of cell labeling, but there was a greater number of labeled cells due to hyperplasia induced by BHA. Because the overall cell proliferation following SO administration was less than that shown by BHA, the researchers suggested that a likely mechanism for SO tumorigenicity in the forestomach was "marginal genotoxicity with strong promotion by increased cell proliferation." They further indicated that at doses that do not induce cell proliferation, no increase in tumors would be expected. Subsequent work (Dalbey et al., 1996) demonstrated increased cell proliferation from gavage administration of 50 mg/kg SO or greater, with a plateau above 250 mg/kg and a NOEL of 20 mg/kg.

These data help interpret the two long-term studies in rats in which SO was administered by gavage at multiple dose levels. Lijinsky et al. (1986) reported no difference in forestomach tumor incidence between 275 and 550 mg/kg/dose (combined papilloma-carcinoma was 50/52 and 50/51 in males and 46/52 and 50/52 in females), while Conti et al. (1988) showed a clear difference between 50 and 250 mg/kg/day (18/40 and 39/40 in males and 11/40 and 38/40 in females). Based on the work of Dalbey et al., doses of 250, 275 and 550 are all above the plateau for increasing cell proliferation and therefore, no difference in response would be expected. No carcinogenicity studies of SO have been conducted at or below the dose the NOEL for increased cell proliferation.

3.1.1.7. SO Mode of Action Summary

In conclusion, SO, when administered by gavage, induces cell damage, cell repair, and increased cell proliferation with marginally detectable DNA adducts in the forestomach; these effects are followed by site of contact tumors in the forestomach, but with no increase in tumors elsewhere. SO is a direct genotoxin *in vitro*, but has a low genotoxic potential *in vivo*. This difference may represent detoxification *in vivo*. The data suggest that SO causes tumors in the forestomach of rodents by a mechanism that is mainly the result of increased cell turnover as a result of the cellular damage caused by high concentrations of SO, but may include direct reaction with DNA as a minor component. The data further suggest that in the absence of cellular damage, SO will not cause increased tumors.

Neither stomach tumors nor stomach tissue damage have been reported in styrene-exposed rodents, confirming the requirement of cellular damage for tumor development from SO gavage.

Humans, with a lesser rate of SO formation from styrene and a more efficient conversion of SO into water soluble metabolites, have been shown to have lower blood levels of SO.

3.2. Non-Genotoxic Mode of Action

The styrene tumor profile suggests a non-genotoxic mode of action by EPA evaluation criteria (EPA, 1996). An increased tumor incidence has been reported in only one species at only one site, and the tumor type is a common one. The increase in tumor incidence was seen only at study termination (24 months) and not at interim sacrifices. The tumors did not result in early mortality in the styrene exposed mice. Furthermore, the tumor response was accompanied by organ toxicity and persistent cell turnover. In addition, a screening assay for genotoxic carcinogens in A/J mice was negative for styrene (Brunnemann et al., 1992). All these aspects of the styrene database support a non-genotoxic mode of action.

The metabolic profile of styrene helps explain species differences in response and supports a non-genotoxic mode of action. The metabolism of styrene in mouse lung Clara cells produces high levels of styrene metabolites that cause Clara cell toxicity. The cellular damage results in reparative responses including increased Clara cell proliferation and hyperplasia of Clara cells. No increased frequency of DNA adducts was found in lung Clara cells of mice exposed to styrene. Rats and humans have a lower metabolism of styrene in lung and more rapid removal of metabolites, thus they do not develop toxicity, increased cell proliferation, hyperplasia, or lung tumors.

Styrene is metabolized to SO, which is genotoxic in *in vitro* experiments and is classified as a probable human carcinogen by IARC (IARC, 1994b). It should be noted, however, that increased tumors were produced in both rats and mice only at the site of contact with SO (forestomach following gavage administration) and that considerable tissue damage preceded tumor formation. Skin application of SO did not result in increased incidence of tumors in the skin or any other site. The lack of systemic tumors from administration of SO supports a non-genotoxic mode of action for SO even though it has been found to be genotoxic. This premise is consistent with the styrene database, which indicates a non-genotoxic mode of action at the site of bioactivation.

3.3. Evaluation of Mode of Action using "EPA Framework"

The analysis in this section is based on the framework and general considerations articulated by U.S. EPA in its Draft Final Guidelines for Carcinogen Risk Assessment (March 31, 2004).

3.3.1. Description of Postulated Mode of Action

The characteristics of the styrene tumor data (increased lung tumors in mice after 2 years, but not 18 months, of exposure by inhalation; by oral exposure lung tumors equivocally increased in mice; no other organs in mice had increased tumor incidence and none were increased in rats exposed by oral or inhalation routes) suggest a non-genotoxic mode of action. The metabolism of styrene in mouse lung Clara cells produces high levels of styrene metabolites, which cause Clara cell toxicity. The cellular damage results in reparative responses including increased Clara cell proliferation and hyperplasia of Clara cells; an increase in lung tumors occurred after 24 months.

Rats and humans have a lower metabolism of styrene in lung and more rapid removal of metabolites, thus they do not develop toxicity, increased cell proliferation, hyperplasia, or lung tumors.

3.3.2. Key Events

In mouse or rat lung, styrene is metabolized nearly exclusively in Clara cells, not in type II cells. The first step is generation of styrene-7,8-oxide (SO), catalyzed primarily by Cyp2F2. Cyp2E1 appears to play a less important role. Using enriched cell fractions, rat and mouse Clara cells produce about the same amount of the S enantiomer of SO per cell, but mice produce five-fold more per cell of R-SO than do rat Clara cells. The production of SO, especially the R enantiomer, is inhibited by 5-phenyl-1-pentyne, an inhibitor of Cyp2F2. Using human lung tissue, very little, if any SO is produced and the S enantiomer predominates. Inhalation of 80 ppm or greater styrene also results in glutathione depletion in mouse, but not rat, lung tissue. In rats, no depletion is seen at levels below 300 ppm and only marginal depletion occurs at higher levels.

Compared to rats, a large proportion of the cells in the terminal bronchioles in mice are Clara cells, the cell with the highest metabolism of styrene. Humans have even fewer Clara cells than rats. In mice, exposure to 40 ppm or greater results in Clara cell cytotoxicity after a single exposure. Continued exposure results in increased Clara cell proliferation, measured by increased BrdU labeling for at least the first two weeks. By thirteen weeks, cellular crowding is seen histologically in the terminal bronchioles. By 12 months, hyperplasia is seen histologically in the terminal bronchioles. With continued exposure, the extent of the hyperplasia increases and it is seen at lower styrene concentrations and a greater percentage of the mice are affected. No toxicity, cell replication or hyperplasia is seen in mouse type II cells. Similarly, no toxicity, increased cell replication, or hyperplasia are seen in rat lung, either for type II or Clara cells. Furthermore, inhibition of styrene metabolism by 5-phenyl-1-pentyne eliminated the cytotoxicity and increased cell replication in mouse Clara cells.

In summary, the metabolism of styrene by Cyp2F2, primarily to R-SO and ring-oxidized metabolites, in mouse lung Clara cells results in cytotoxicity, increased cell proliferation, cell crowding, hyperplasia and eventually lung tumors. Rat lung tissue, with fewer Clara cells and much less Cyp2F2 in those Clara cells, does not produce toxic quantities of styrene metabolites, as evidenced by lack of increased cell proliferation, absence of histopathologic effects in lungs and no increase in lung tumors. In humans, with barely detectable levels of Cyp2F1 (the human equivalent of Cyp2F2) and no measurable metabolism of styrene by Cyp2F1, even less toxic metabolites are produced than in rats and those which are produced or diffuse into lung from the blood stream are removed more efficiently by epoxide hydrolase. Thus, humans have even less likelihood of developing styrene-induced lung cytotoxicity, increased cell proliferation or lung tumors than rats.

3.3.3. Strength, consistency, specificity

Mice have a much greater number of Clara cells than do rats, which have a much greater number than humans. In addition, mouse Clara cells have much more Cyp2F2 and produce much more

R-SO and ring-oxidized metabolites than do rat Clara cells; human lung cells have barely detectable levels of Cyp2F1 and no detectable metabolism of styrene. Thus mice have the greatest number of target cells for styrene toxicity and those target cells have the greatest capacity to produce toxic metabolites of styrene. Increased production of SO, especially R-SO, in mouse lung has been shown by the use of microsomes and isolated cell fractions. Using ip injection, R-SO was shown to be more pneumotoxic (increased GGT and LDH in BALF) than S-SO, consistent with the increased production of R-SO in mouse lung. Further, ring-oxidized metabolites from 4-vinylphenol are at least 5-fold more toxic in mouse lung than metabolites derived from R-SO. Thus, styrene metabolite-induced toxicity in mice occurs in the organ where increased tumors are seen.

Styrene toxicity in mice occurs in 3 organs: liver (acute necrosis, but cells replaced with resistant hepatocytes), nasal olfactory mucosa (chronic cytotoxicity, limited cellular replacement, cells replaced with respiratory-like cells), and lung (chronic cytotoxicity, rapid cellular replacement in kind, hyperplasia). These are the organs highest in Cyp2F2. In both lung Clara cells and nasal olfactory mucosa, metabolism of styrene per cell is markedly greater than that in mouse hepatocytes and the formation of R-SO is preferred even more than in mouse hepatocytes. Toxicity in both olfactory mucosa and Clara cells is prevented if Cyp2F2 is inhibited by 5-phenyl-1-pentyne. In rat lung and liver, with very little Cyp2F2, styrene is metabolized via Cyp2E1. Rat nasal olfactory tissue contains a moderate amount of Cyp2F4, in addition to CYP2E1. In rat lung and liver the ratio of R-SO to S-SO is less than 1, but in rat nasal olfactory tissue, it is about 3. Thus the only rat tissue that contains measurable amounts of Cyp2F4 (rat olfactory epithelium) is the only tissue that produces a high proportion of R-SO and is the only rat tissue to display styrene induced cytotoxicity.

The induction of lung tumors in mice does not appear to be related to the formation of styrene-DNA adducts because:

1. The formation of DNA adducts following styrene exposure is low in rats, mice and humans (<1 per 10^7 nucleotides).

2. No unique styrene-DNA adducts have been found in mouse lung.

3. The level of DNA adducts in mouse lung is not greater than in mouse liver.

4. The level of DNA adducts in mice is not greater than in rats.

The induction of lung tumors in mice appears to involve mouse lung-specific metabolism of styrene, and is independent of the blood level of SO. In the chronic inhalation studies, the blood levels of SO in rats exposed to 1000 or 200 ppm styrene were 170 and 47 µg/l (male and female averaged), respectively, while mice exposed to 160 ppm had blood SO levels of 27 µg/l. It should be noted that in the chronic study 11 of 20 mice exposed to 40 ppm for 73 weeks had no detectable blood SO and none of those exposed to 20 ppm had detectable blood SO; yet Clara cell toxicity was seen in mice exposed to 20 ppm and greater. Because exposure concentrations in rats (up to 1,000 ppm) that did not cause increased lung tumors resulted in higher blood levels of SO than exposure levels in mice that did result in increased lung tumors, and because effects

were seen in mouse lung following exposure to 20 ppm where SO was not detected in the blood, effects seen in mouse lung are not likely the result of the influx of SO from the bloodstream.

3.3.4. Dose/Response

The non-neoplastic key events of styrene in mouse lung occurred at the same exposure concentrations that resulted in increased lung tumors. Decreased staining of the Clara cells (an indicator of cytotoxicity) was reported in 50-70% of the mice exposed to 20 ppm for 12, 18 or 24 months and in more than 80% of those exposed to 40, 80, or 160 ppm. Increased cell proliferation has been reported at concentrations of 40 ppm or greater (20 ppm has not been examined). Bronchiolar hyperplasia was seen in a few mice exposed to 40 ppm for 12 months and in most mice exposed to 80 or 160 ppm; by 24 months bronchiolar hyperplasia was seen in up to 40% of the mice exposed to 20 ppm and in more than 75% of those exposed to 40, 80 or 160 ppm. Because the non-neoplastic effects of styrene are seen in nearly all mice at exposure levels of 40 ppm and above, it is not surprising that there is not a clear dose-response in tumor formation.

3.3.5. Temporality

In mice, increased lung tumors were seen only at the end of the study. Short-term studies and interim assessments during the chronic study indicate that all the described precursor lesions preceded the formation of lung tumors. Single exposures to styrene result in the production of large amounts of metabolites in mouse lung and produce cytotoxicity following a single exposure. After a few exposures, increased repair is measurable as increased labeling of Clara cells. Continued exposures of a few weeks result in Clara cells that stain less well with eosin and crowding of Clara cells, which could be described as an increased number of cells but not extra layers of cells. Sometime between 13 and 52 weeks, this increase in cells become great enough that extra layers of cells are detectable; this is described as hyperplasia of the terminal bronchioles. With continued exposure, the hyperplasia extends into the alveolar ducts. Lastly between 18 and 24 months increased lung tumors appear.

3.3.6. Plausibility and Coherence

The lung tumors found in styrene exposed mice fit with a non-genotoxic mode (single species, single organ, late occurring). In addition, there is no support for genotoxicity from styrene in mouse lung (no increase in CAs in mouse lungs, very low levels of non-specific DNA adducts in mouse lungs, negative in A/J mouse screening assay), as well as no increase in HGPRT mutations in hamster lung V79 cells. Lack of lung tumors as a factor contributory to death, except in a few high exposure females, provides additional support for a non-genotoxic mode of action.

Styrene causes mouse-specific lung cytotoxicity due to mouse-specific metabolism of styrene. Cytotoxicity, with compensatory cell replication, often results in increased tumor incidence; this is believed to be due to the increased probability of fixing spontaneous mutations in dividing cells because of the increased cell replication and the resulting decreased time for repair of mutations.

Depletion of GSH occurs in mouse lungs at levels that resulted in increased tumor formation. Depletion of GSH results in an inability to detoxify normal oxidative compounds inhaled or generated during normal metabolism. These oxidative compounds can cause increased DNA adducts, such as 8-oxyguanosine, which lead to DNA strand breaks and potentially to increased tumor formation.

The data are also consistent with Cyp2F2 catalyzed metabolism of styrene to largely R-SO. In mice, lung Clara cells and nasal tissue have large amounts of Cyp2F2, produce much more R-SO than S-SO, and develop cytotoxicity. Inhibition of Cyp2F2 by 5-phenyl-1-pentyne in mice for three days eliminated the nasal and Clara cell toxicity from exposure to 160 ppm styrene. In rats, Cyp2F2 has been detected only in nasal tissue; not surprisingly, then cytotoxicity is seen in the olfactory region of rats, as well as in mice. Rat Clara cells have very limited amounts of CYP2F2, produce more S-SO than R-SO, and do not develop cytotoxicity.

A number of aromatic chemicals are metabolized by Cyp2F2 in mouse lung, cause mouse lung Clara cell cytotoxicity and mouse lung tumors. Chemicals such as naphthalene and coumarin show a similar pattern of metabolism and effects in mouse lung as seen with styrene.

The current data indicate a greater production of R-SO in tissues where cytotoxicity is seen; however, very low levels of bound metabolites, and specifically low levels of DNA adducts, suggest that the cytotoxicity may be caused by further metabolism of R-SO or by ring-oxidized metabolites.

In sum, the styrene data fit with a non-genotoxic mode of action. Lack of genotoxic reactions in mouse lung tissue and lack of tumor response in tissues other than mouse lung support this conclusion. Mouse lung specific metabolism and cytotoxicity of styrene and elimination of the cytotoxicity by inhibition of the metabolism of styrene provide further support for the non-genotoxic mode of action.

3.3.7. Other Modes

The default assumption of tumor formation involves a genotoxic mode of action. Some positive genotoxic findings have been reported for styrene. Further, styrene is metabolized to SO, which consistently has demonstrated genotoxic effects in *in vitro* studies. SO reacts with DNA to form adducts; DNA adducts have been demonstrated in rats, mice and humans exposed to styrene and/or SO. Thus there is evidence of a genotoxic material reaching the target organ and reacting with DNA in the target organ, which could be interpreted to mean that the default assumption is correct. The data also demonstrate the presence of that genotoxic agent in human blood and reaction of that agent with human (lymphocyte) DNA. However, the styrene database presents important inconsistencies with the default assumption.

In general, genotoxic agents cause increased tumors in more than one species and/or at more than one site. Frequently there is good evidence of increased malignant tumors at multiple sites. None of these database attributes fit the styrene database. Increased malignant tumors (lung) were found only in high exposure female mice and only in one of 5 studies. In three of the five studies increased benign (and combined benign and malignant) lung tumors were identified as the only

site, but the increase was not consistent as to the sex affected. No tumor sites were increased in the 8 rat studies. The overall body burden of SO in rats and mice in the chronic studies does not support an SO-mediated genotoxic mode of action. SO levels, as measured by blood SO, were higher in at least some of the rat studies (up to 1,000 ppm or 1,000 mg/kg/day styrene) than in the mouse studies where increased lung tumors were observed. This suggests that target organ metabolism, not circulating blood levels of SO, is important for tumor formation. The metabolism of styrene to SO occurs to a greater extent in mouse lungs than in rat lungs and to a greater extent in mouse lung than in mouse liver, suggesting that mouse lungs are more likely to sustain genotoxic reactions from SO than are rat lungs or mouse liver. However, the limited data available indicate that styrene is not genotoxic in mouse lung tissue; i.e., no increase in CAs in mouse lung tissue and negative in A/J mouse screening assay. In addition, styrene exposure results in very low levels of DNA adducts (<1 per 10^7 nucleotides) in mouse lungs, which are not unique to lung tissue and do not occur at a greater frequency than in mouse liver or rat lung.

Thus while styrene and SO have some genotoxic properties, the overall evidence does not support a genotoxic mode of action for the induction of mouse lung tumors.

3.3.8. Conclusions

The mode of action data for styrene fit with a non-genotoxic action as a result of the high metabolism of styrene by Cyp2F2 in mouse lung Clara cells. Data have been presented which show the specificity of the metabolism in the target cell, cytotoxicity in the target cell, increased cell replication in the target cell, leading to hyperplasia and increased tumors at the end of the study. Inhibition of the styrene metabolism eliminated the cytotoxic effects of styrene exposure in mouse lung (and nasal tissue). Styrene has been shown to have a weak genotoxic potential, and measures of genotoxicity in mouse lung are negative.

Although the cell of origin for the styrene-induced mouse lung tumors has not been established, no difference in tumors between the styrene-exposed and control mice has been seen, further suggesting a "promotional" type increase in spontaneous tumors.

Based on the tumor data for styrene, evidence of cytotoxicity and cell proliferation dependent on the mouse lung specific metabolism of styrene, and the lack of evidence of a genotoxic mode of action, it is concluded that styrene induces tumors in mouse lung tissue by a non-genotoxic mode of action.

3.3.9. Human Relevance

The carcinogenic potential of styrene has been studied in a number of cohorts of workers exposed to styrene in the workplace. No consistent evidence of an increased risk of cancer from styrene exposure is found in these studies. In the reinforced plastics industry, past exposures may have consistently exceeded 100 ppm. Environmental exposures are no greater than the low parts per billion (ppb) range, and most are below 1 ppb.

The sharp contrast between the positive mouse tumor data and the negative rat and human data is not surprising in light of the mode of action for the styrene-induced mouse lung tumors.

Exposure of mice to styrene results in metabolism by a number of cytochrome P450 isoforms. In lung and nasal tissue, Cyp2F2 appears to be the most important, leading to a high production of R-SO and to subsequent metabolites. Cytotoxicity is seen in both tissues. In nasal tissue, damaged olfactory cells are replaced with respiratory-like cells that appear to be resistant to effects from styrene exposure. In lungs, Clara cells are damaged and rapidly replaced by more Clara cells which, in turn, are damaged and replaced, eventually leading to an increase in lung tumors.

Rat lung Clara cells have much lower levels of Cyp2F2, which leads to a significantly lower production of R-SO in rat lung; this explains why exposure of rats to much higher levels of styrene does not result in cytotoxicity or increased lung tumors. Humans have even fewer Clara cells than rats and those Clara cells have much less Cyp2F1 (human equivalent of Cyp2F2) than rats. Limited human studies show little if any production of SO from styrene by Cyp2F1 and little if any SO production by microsomes prepared from human lung tissue. Thus, neither cytotoxicity nor lung tumors are expected in humans exposed to environmental or occupational exposures to styrene. This is supported by a lack of styrene-related increased lung tumors in the cohort mortality studies of workers exposed to styrene.

Because the tumorigenicity of styrene is related to its metabolism by Cyp2F2, children are not expected to be more sensitive to styrene than adults since P450 activity is not greater in children than in adults.

4. Conclusion

Section 301 (b) (4) of the Public Health Services Act directs the Secretary of the Department of Health and Human Services to publish a report which contains a list of all substances: (1) that are either known to be human carcinogens in humans or reasonably be anticipated to be human carcinogens, and (2) to which a significant number of persons residing in the United States are exposed. The criteria that NTP applies to determine whether a substance is carcinogenic is reported at 64 Fed. Reg. 15983 (April 2, 1999).

Under these criteria, NTP assesses whether a substance is a known human carcinogen based on sufficient evidence of carcinogenicity from human studies that indicates a causal relationship between exposure to the substance and human cancer.

Overall, the human data from 12 cohort mortality studies do not present any convincing evidence that styrene causes increased cancer in humans. Among more than 61,000 reinforced plastics workers in three studies, which represent the workers most highly exposed to styrene, there is no consistent increase in any cancer, specifically with lymphatic and hematopoietic cancers. In the Kolstad study, increased leukemia was found among short-term, but not long-term workers; in addition, it is not possible from this study to determine if any of the leukemia cases were actually exposed to styrene and, if so, to what levels. Increased cancers in monomer, polymer and SBR workers are not attributed to styrene. While there are some increased cancer deaths among workers in industries that use styrene, there is no evidence that these increases are caused by styrene exposure. Thus, the existing human studies do not constitute sufficient evidence on which NTP should include styrene in the Report on Carcinogens.

NTP also evaluates whether a substance is reasonably anticipated to be a human carcinogen based on limited evidence of carcinogenicity in humans and/or sufficient evidence of carcinogenicity in experimental animals. Sufficient evidence in animals is demonstrated by positive carcinogenicity findings in multiple species, or at multiple tissue sites, or by multiple routes of exposure, or to an unusual degree with regard to incidence, site or type of tumor or age at onset.

Again, a review of the human and animal data for styrene do not fall within these criteria. Although there are some increased incidences of cancer in studies of workers exposed to styrene, there is no evidence that styrene is causal in those increases, and exposure to other chemicals or other factors may be causal. Therefore, SIRC does not concur with the IARC conclusion of limited evidence in human data. We agree with IARC that the animal data provide only limited evidence of carcinogenicity and that the extensive mode of action data indicates that the mouse lung tumors are the result of cytotoxicity caused by *in situ* metabolism of styrene and are not relevant for human risk assessment. Since the last IARC review, fairly strong evidence has emerged to indicate that ring-oxidized products of styrene are responsible for the cytotoxicity and styrene-7,8-oxide plays a minor role, if any. These ring-oxidized products are also generated by CYP2F2 in the mouse lung. In contrast, the metabolism of styrene in the human lung is extremely low and no activity of the human CYP2F1 to styrene has been detected. This provides further evidence that the mouse lung tumors are not relevant for human risk assessment.

Based on the preceding review of the data, SIRC believes that styrene does *not* meet NTP's criteria for inclusion in the Report on Carcinogens. SIRC appreciates the opportunity to provide comments for NTP's assessment of styrene, and would be pleased to discuss the studies referenced in this document. For any questions, or for more information, please contact SIRC's executive director, Jack Snyder, at (703) 741-5010 or at SIRC@styrene.org.

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